

RAPID MULTIPLE PANEL OF BIOMARKERS IN LABORATORY BLOOD TESTS FOR TIA/STROKE

BACKGROUND OF THE INVENTION

Relation to Prior Applications

This application claims priority under 35 U.S.C. § 119(e) to United States Provisional Application No. 60/301,297, filed June 27, 2001, and under 35 U.S.C. § 120 to United States Utility Application No. 09/632,749, filed August 4, 2000 (currently pending), of which this application is a continuation-in-part.

Field of the Invention

The present invention relates generally to the diagnosis, management and therapy of central nervous system disorders such as stroke, transient ischemic attack, and traumatic brain injury. In particular, the invention relates to methods and kits for evaluating these central nervous system disorders, in order to better respond to episodes of focal cerebral ischemia, and to best manage the risk associated with future acute incidences.

Background Information

Stroke or "brain attack" is clinically defined as a rapidly developing syndrome of vascular origin that manifests itself in focal loss of cerebral function. In more severe situations, the loss of cerebral function is global. A stroke occurs when the blood supply to the part of the brain is suddenly interrupted (ischemic) or when a blood vessel in the brain bursts, spilling blood into the spaces surrounding the brain cells (hemorrhagic). The symptoms of stroke are easy to spot: sudden numbness or weakness, especially on one side of the body; sudden confusion or trouble speaking or understanding speech; sudden trouble seeing in one or both eyes; sudden trouble walking; dizziness; or loss of balance or coordination. (National Institute of Neurological Disorders and Stroke, 2001). Stroke is the most common devastating neurologic disease in the world, and the third leading cause of death in world after heart disease and cancer. Despite recent progress understanding stroke mechanisms, stroke management is still not optimal for a number of reasons.

The importance of promptly diagnosing a stroke after symptoms appear cannot be overstated. Delays in diagnosis and medical intervention beyond 3 hours after stroke onset may contribute to clinical deterioration and disability. An early diagnosis enables doctors to

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more effectively choose the emergency intervention such as anti-platelet or/and neuroprotective therapy, and also to make better prognoses of disease outcome. Successful treatment of stroke requires rapid state diagnosis. The delay in achieving an accurate and certain diagnosis wastes the limited amount of time available in which the brain can respond to reperfusion, and significantly increases the risk of hemorrhage after most of the permanent injury has occurred (Marler J.R. *Annl. Emergency Med.* 1999, 33: 450-451).

Unfortunately, however, many people who have a stroke either do not seek immediate medical care or suffer from delays in medical care even in countries where stroke care is advanced, such as the United States and Europe (Alberts MJ, Hademenos G, Latchaw RE, et al. *JAMA* 2000; 23:3102-3109). Several clinical criteria can be employed to diagnose whether a patient is having a stroke, but even all these criteria do not always allow one to differentiate the episode from other disorders, such as epilepsy, syncope, and migraine (Toole JF. *Cerebrovascular Disorders.* 1999 . Lippincott, Williams & Wilkins, New York, 5th Ed., 542 p). Moreover, progressing stroke is only partially predictable based on clinical and neuroimaging data that is currently available to neurologists.

Transient ischemic attack (TIA) is a short-lived episode of focal neurologic deficit which often precedes the cerebral infarction of a stroke. It occurs when the blood supply to part of the brain is briefly interrupted, and is typically accompanied by permanent brain damage (albeit less severe damage than normally results from a stroke). TIA symptoms, which usually occur suddenly, are similar to those of stroke but do not last as long. Most symptoms of a TIA disappear within an hour, although they may persist for up to 24 hours. Symptoms can include: numbness or weakness in the face, arm, or leg, especially on one side of the body; confusion or difficulty in talking or understanding speech; trouble seeing in one or both eyes; and difficulty with walking, dizziness, or loss of balance and coordination. (National Institute of Neurological Disorders and Stroke, 2001). Patients who have suffered a TIA have 9.5 times greater risk of having a future stroke than those who have not had a TIA, and about one third of patients who suffer a TIA will have an acute stroke in the future. (American Stroke Association, 2001). However, because the symptoms of TIA are short term, many patients do not recognize the event as a TIA or perceive the event as a warning of a potentially impending stroke.

Standard treatments to reduce the risk of future stroke include the use of antiplatelet agents, particularly aspirin. People with atrial fibrillation (irregular beating of the heart) may be prescribed anticoagulants. The most important treatable factors linked to TIAs and

stroke are high blood pressure, cigarette smoking, heart disease, carotid artery disease, diabetes, and heavy use of alcohol. Lifestyle changes can often be implemented to reduce these factors. However, it is necessary to diagnose the TIA as a warning sign of impending stroke before such treatments can be administered. Therefore, a laboratory blood test to detect TIA or stroke, or the risk of suffering a TIA or stroke in the future, would be of tremendous benefit.

During the past 5 years a number of molecular and immunochemical assays have been evaluated for clinical use in neurology. (Schenone A. et.al. *Current Opinion in Neurology*. 1999, 12: 603-604; Honnorat J. *J.Neurol. Neurosurg. Psychiatry*. 1996, 61:270-278). At present, the Thrombogene V and two Thrombx tests are available for diagnosing stroke/thrombosis from Athena Diagnostic. These tests evaluate the frequent deep vein thrombosis and hypercoagulation states of patients to evaluate the need for intravenous anticoagulant therapy. The Thrombogene V test detects the Factor V Leiden mutation by Polymerase Chain Reaction (PCR) in the blood of patients. The other two tests monitor changes of different blood coagulation markers: antithrombin III protein C, factor IX, and anticardiolipin antibodies (IgG, IgM, IgA) by use of ELISA technique. These tests thus reveal the hypercoagulation state as a result of a thrombotic events, such as stroke stroke.

Stroke can be related to different types of venous thromboembolisms, which are common disorders with considerable morbidity and potential for mortality (Anderson,D.;Wells,P. *Cur.Opinion in Hemat.* 2000, 7: 296-301). The biochemical marker: D-dimer, a breakdown product of a cross-linked fibrin blood clot that indicates the occurrence of plasmin mediated lysis of cross-linked fibrin, has been extensively evaluated for use in diagnostic tests for indicating acute venous thromboembolism. Indeed, a fully automated, semi-quantative latex agglutination assays that uses turbimetric or agglutination endpoints has been developed that provides results within 20 minutes with sensitivity between 89% and 95% (Roussi J.;Bentolila L.;Contribution of D-dimer determination in the exclusion of deep venus thrombosis in spinal cord injury patients. *Spinal Cord* ,1999; v.37: p.548-552). Unfortunately, however, the presence of D-dimer may also be increased in other settings that result in fibrin generation, including recent surgery, hemmorrhage, trauma, cancer, and pregnancy (Anderson DR., Wells PS.; *Thromb.Haemost.*; 1999; 82:878-886).

However, the foregoing tests do not elucidate upon the TIA/stroke mechanisms that are actually responsible for the damage associated with neurotoxic molecular events. It is

necessary to find out specific and sensitive biomarkers which could be helpful to recognize initial brain damage and which could help to choose not only the appropriate anticoagulant treatment, but also emergency or regular neuroprotective therapy.

It is well known that two of the three leading causes of death, namely cardiovascular diseases and stroke, are the end result of atherosclerosis. Thus, it is not surprising that several biochemical markers implicated in thromboembolic processes are also reported to be associated with stroke and stroke risk. Among these are homocysteine, cholesterol and LDL (Cerebrovascular Disorders ed. by J.E.Toole. Lippincott Williams & Wilkins. 1999, pp.34-35), which are also classified as risk factors to cardiovascular and cerebrovascular diseases. (Hankey GJ., and Eikelboom JW. Lancet. 354: 407-413 (1999). Approximately one fourth of patients with symptomatic atherosclerosis have elevated plasma homocysteine levels caused by various factors.. High levels of homocysteine may run in families with increased susceptibility to heart attack and stroke (Graham I. J.Ir.Call.Phys.Surg. 1995; 24: 25-30). Elevated plasma homocysteine may be a causal and modifiable risk-factor for ischemic stroke, but the results of previous studies have been conflicting (Deulofeu VNR, Chamorro A, Piera C. Med Clin (Barc). 1998; 110: 605-608; Yamamoto T, Rossi S, Stiefel MF, Doppenberg E, Zauner A, Bullock R, Marmarou A. Acta Neurochir.Suppl.(Wien) 1999; 75:17-19).

The neurotoxic effect of excitatory amino acids (glutamate, aspartate) in the brain has also been well documented. The results of this work show a correlation between glutamate content in the blood and the severity of acute ischaemia (Castillo J, Dávalos A, Naveiro J, Noya M. Stroke 1996, 27:1060-1065; Castillo J, Dávalos A, Noya M. Lancet. 1997; 349:79-83). Cerebral damage and its association with progressing stroke has been attributed to increased glutamate release, or low glutamate reuptake, both in animals and in humans (Dávalos A, Castillo J, Serena J, Noya M. Stroke 1997; 28:708-710).

However, only 56% of patients with progressing stroke are reported to have high glutamate content in their blood serum (Dávalos A., Toni D., Iweins F., *et al.*, 1999, 30: 2631-2636). Moreover, even though glutamate is considered the strongest biochemical predictor of progressing stroke (Dávalos A, and Castillo J. In Book: *Cerebrovascular Disease*. Current Med.Inc.: Philadelphia. 2000 Chapter 16, pp.169-181), this marker remains non-specific for TIA. Glutamate changes have also been observed in the blood of patients with epilepsy and other nervous system disorders (Meldrum BS. J.Nutrition. 2000, 130:1007S-1015S).

Glutamate receptors are divided into two main groups: ionotropic and metabotropic. The ionotropic neuroreceptors are ligand-gated ion channels that are subdivided into NMDA, AMPA and kainate receptor subtypes. There are four NR2 subunits: NR2A, NR2B, NR2C and NR2D, which is responsible for Ca^{2+} -permeability regulation. NMDA receptors can be modified by ischemia, resulting in changes of ion permeability and/or ion selectivity.

In a similar vein, several researchers have reported an increase in NMDA receptor synthesis, the appearance of high levels of receptor antigen, and the generation of autoantibodies to the receptors during the initial stages of cerebral ischemia (Gusev et al., J.Neurol.& Psych.1996, 5:68-72; Dambinova et al. J.Neurol.Sci. 1997, 152:93-97; Dambinova et al. J.Neurochem. 1998, 71: 2088-2093). Acting on this research, one

company developed a laboratory kit (cerebral ischaemia (CIS)-test) that detects autoantibodies to the N-terminus domain of the NR2A subunit in the blood of patients with TIA or stroke (Gusev E.I., Skvortsova VI, Alekseev AA, Izykenova GA, Dambinova SA. S.S Korsakov's J.Neurol.& Psych. 1996; 5:68-72). The N-terminus domain of the NR2A subunit of NMDA receptors was selected as the immunoreactive epitope on the basis of molecular biological and experimental studies showing that this epitope is the most immunoreactive region of the receptor (Dambinova SA, Izykenova GA. J.High Nervous Activity. 1997; 47:439-446).

More recently, researchers have reported a correlation between the effectiveness of a stroke treatment regimen and the levels of autoantibodies to the NR2A and NR2B subunits of NMDA. In particular, these researchers have reported increased titers of autoantibodies to the NR2A and NR2B subunits of NMDA in the blood of patients severely affected by stroke, and a reduction of the autoantibodies, accompanied by an improvement in neurological function, during therapy by glycine - a non-specific agonist of NMDA receptors (Gusev et.al. Cerebrovascular Diseases. 2000, 10: 49-60). Patients that responded positively to glycine had lower autoantibody titers than patients who were not treated, and had levels of autoantibodies that were close to the levels of autoantibodies in control subjects.

Unfortunately, the use of NR2A and NR2B autoantibodies in the diagnosis of stroke or TIA does not provide a real-time assessment of the damage being done by a stroke or TIA. Rather, because of the time the immune system requires to mount an immune response, and to generate NR2A and NR2B autoantibodies, methods that test for these antibodies at best provide a delayed assessment of the extent and severity of stroke or TIA.

Investigators from Canada (Hill M.D., Jackowski G., Bayer N., Lawrence M., Jaeschke R. Can.Med.Assoc.J. 2000, 163: 1139-1140) have proposed a new diagnostic laboratory assay for differentiating stroke subtype. They designed a preliminary prospective cohort study to test a panel of biochemical markers (neuron-specific enolase[NSE], myelin basic protein [MBP], S-100 [beta] protein and thrombomodulin [Tm]) in blood samples from patients with acute ischemic stroke. These markers were chosen because they cover important cellular components of the brain that might be damaged in acute stroke. The 4 biochemical markers were assayed using a standard ELISA technique.

For stroke, 3 hours is an outside limit for administering appropriate therapies. The focus must change from extensive evaluation before any action to a well-planned acute emergency therapy developed using an appropriate diagnostic strategy. Every future advance to improve the outcome after TIA /stroke will depend on a fast initial response-within minutes and not hours (Marler J.R. *Annl. Emergency Med.* 1999, 33: 450-451). Therefore, it is especially important to develop a fast and simple method (within one hour) of detecting brain and blood biomarkers capable of recognizing the initial processes of TIA/stroke before irreparable ischemic damage ensues.

Therefore, it is an object of the invention to provide biochemical methods and kits for diagnosing central nervous system disorders such as TIA and stroke.

It is still another object of the present invention to provide methods of diagnosing stroke using biochemical markers that distinguish between hemorrhagic and ischemic stroke.

It is another object of the present invention to provide rapid biochemical methods and kits for diagnosing TIA and stroke, to provide real-time assessments of TIA or stroke, within a window of time that permits effective therapeutic intervention.

It is another object of the present invention to provide rapid and inexpensive biochemical methods and kits for diagnosing TIA and stroke, which can be performed at

frequent intervals to monitor the progression of a TIA or stroke, or the effectiveness of therapy administered against TIA or stroke.

Still another object of the present invention is to provide diagnostic methods and kits for assessing the risk of incurring a TIA or stroke, and for monitoring the remission of risk factors for TIA or stroke.

Still another object of the invention is to provide a panel of rapid multiple panel of biomarkers for assessing the nature, severity and progression of TIA or stroke, and thereby to enable a more effective selection of intervention therapy.

SUMMARY OF THE INVENTION

It has unexpectedly been discovered that levels of circulating NMDA receptor proteins or fragments thereof can be assessed using diagnostic kits and processes, and that levels of these proteins or fragments can be used to clinically evaluate patients suffering from ischemic central nervous system disorders such as stroke or TIA. When analyzed in combination with other biomarkers for stroke and TIA, such as the thromboembolic marker homocysteine, or the excitatory amino acid glutamate, these proteins can diagnose the existence of a stroke with remarkable accuracy (generally greater than 89%). In contrast, the efficacy of single parameters for early diagnosis of stroke is 58% for glutamate, 66% for homocysteine, and 79% for NMDA receptors. The rapid evaluation of these neural ischemic biomarkers in an emergency room setting will greatly enhance the confidence of physicians when diagnosing stroke or TIA, and significantly improve the speed at which therapy against the stroke or TIA can be administered.

The biomarkers also yield extensive evidence about the nature of the stroke or TIA and the type therapy which should be administered. For example, the respective levels of biomarkers can be evaluated to determine whether the patient is suffering an ischemic or hemorrhagic stroke, or whether the patient is suffering from a traumatic brain injury. The data from the biomarkers can also be used to monitor or evaluate the progression of the ischemic episode, as well as the damage that has resulted as a consequence of the ischemia. High levels of all parameters reflect the neurological deficit and may be also used for prognosis of disease outcome. Moreover, a relationship has been observed between the respective levels of the biomarkers and the degree of thromboembolic and neurotoxicity in brain processes under the stroke. Once again, these relationships can be put to extensive use when evaluating the choice of emergency therapy in short time frames, such as anti-platelet

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and neuroprotective therapy. The data can be used independently of other diagnostic strategies, but preferably forms an integral part of a comprehensive diagnostic strategy employing conventional diagnostic techniques.

The data obtained from the NMDA biomarkers, especially when combined with data from other biomarkers such as glutamate and homocysteine, can also be used to monitor the efficacy of a treatment regime. It has surprisingly been found that the NMDA biomarkers provide real time evidence of neurotoxicity, and that reductions in levels of circulating NR1A or NR2A NMDA receptors or fragments thereof correspond well with reductions in neurotoxic mechanisms. By obtaining data at appropriate intervals using rapid laboratory techniques such as latex agglutination, one is able to monitor the progression of the episode in response to the therapeutic regime.

A latex agglutination technique has also been developed which dramatically increases the speed of diagnosis obtained by the methods of this invention, and thereby improves the effectiveness of the methods in emergency-room settings. The technique can be adapted for use in the detection of NMDA receptors, homocysteine, glutamate, or any other suitable biomarker against central nervous system disorders. Using the latex agglutination technique, one is able to provide real-time biochemical diagnosis and monitoring of TIA/stroke patients (within about 30 minutes), and thereby dramatically improve the effectiveness of response to TIA/stroke. This is surprising because these biomarkers are naturally occurring and, in contrast to viruses for which latex agglutination methods were originally developed, show much lower strengths of association with their corresponding antibodies.

This semi-quantitative method gives reliable data quickly in a format that is simple for interpretation. Surprisingly, the technique shows greater accuracy than even well established methods based upon HPLC and ELISA. The application of the latex agglutination technique to the analysis of brain biomarkers for stroke will decrease the cost of analysis, provide the opportunity to monitor real-time progress of a treatment procedure, and allow physicians to determine the efficacy of medication administered in the treatment of TIA or stroke.

The methods of the present invention also can be employed in a non-emergency setting, when evaluating the risk that an individual will suffer a stroke or TIA. In addition, based upon results showing an increased risk of suffering TIA or stroke, prevention therapy

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can be administered, and the effectiveness of the therapy monitored using the methods of the present invention.

The invention also relates to indirect methods for measuring levels of NR2A and NR2B NMDA receptor proteins or fragments thereof. Thus, analytical techniques can be used to evaluate indirect measures of NR2A and NR2B NMDA receptor proteins or fragments thereof, such as autoantibodies specific for the proteins, or cDNA that encodes for the proteins.

Additional advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Examples included therein. Before the present methods and techniques are disclosed and described, it is to be understood that this invention is not limited to specific analytical or synthetic methods as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

Definitions and Use of Terms

As used in this specification and in the claims which follow, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a fragment" includes mixtures of fragments, reference to "an cDNA oligonucleotide" includes more than one oligonucleotide, and the like.

An analogue of a protein, peptide, or polypeptide means a protein, peptide, or polypeptide that contains one or more amino acid substitutions, deletions, additions, or rearrangements. For example, it is well known in the art of protein biochemistry that an amino acid belonging to a grouping of amino acids having a particular size or characteristic (such as charge, hydrophobicity, and hydrophilicity) can often be substituted for another

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amino acid without altering the activity of the protein, particularly in regions of the protein that are not directly associated with biological activity. Thus, an analogue of an NMDA receptor or fragment thereof is useful in the present invention if it includes amino acid substitutions, deletions, additions or rearrangements at sites such that antibodies raised against the analogue are still specific against the NMDA receptor or fragment.

Preferably, an NMDA analogue has at least 80%, 85%, 90%, or 95% amino acid identity with naturally occurring NMDA. Amino acid identity is defined by a analogy comparison between the analogue and naturally occurring NMDA. The two amino acid sequences are aligned in such a way that maximizes the number of amino acids in common along the length of their sequences; gaps in either or both sequences are permitted in making the alignment in order to maximize the number of common amino acids. The percentage amino acid identity is the higher of the following two numbers: (1) the number of amino acids that the two polypeptides have in common with the alignment, divided by the number of amino acids in the NMDA analogue or fragment thereof, multiplied by 100, or (2) the number of amino acids that the two polypeptides have in common with the alignment, divided by the number of amino acids in naturally occurring NMDA or fragment thereof, multiplied by 100.

NMDA derivatives, and derivatives of NMDA fragments, include naturally occurring NMDA and NMDA analogues and fragments thereof that are chemically or enzymatically derivatized at one or more constituent amino acids, including side chain modifications, backbone modifications, and N- and C- terminal modifications, by for example acetylation, hydroxylation, methylation, amidation, phosphorylation or glycosylation. The term also includes NMDA salts such as zinc NMDA and ammonium NMDA.

A protein or peptide is measured "directly" in the sense that the protein or peptide is itself measured in the biological sample, as opposed to some other indirect measure of the protein or peptide such as autoantibodies to the protein or peptide, or cDNA associated with the expression of the protein or peptide.

The term "antibody" is intended to be synonymous with "immunoglobulin." As used herein, the term "antibody" is meant to include both the native antibody, and biologically active derivatives of antibodies, such as, for example, Fab', F(ab')₂ or Fv as well as single-domain and single-chain antibodies. A biologically active derivative of an antibody retains the ability to bind antigen.

General Discussion

The present disclosure describes diagnostic and therapeutic applications that result from the realization that genetic or accidental increase of NMDA receptors synthesis in the brain reflects a neurological ischemic deficit, and may be used for early diagnoses of stroke or TIA. NMDA receptors that are abnormally expressed in the brain are quickly metabolized and, following penetration of the blood brain barrier, these metabolic destruction products enter the circulatory system. The immune system recognizes these peptides and protein fragments as foreign antigens and responds by generating autoantibodies to them.

In one aspect of the present invention, the correlation between increased NMDA receptor synthesis, and the appearance of high levels of the receptors in blood sera of individuals during the initial stages of cerebral ischemia, is used for diagnostic and therapeutic applications. Experiments in rats with focal ischemia have demonstrated that NR2A mRNA expression in the cortex and hippocampus can be measured within two hours of the onset of the ischemic episode, and thus provide an opportunity for real time measurement of ischemic processes and damage resulting therefrom. At the same time, meaningful expression of NR2C and NR2D mRNA is not observed in brain structures that showed no changes in NR1 mRNA expression in rat ischemic brain. These changes in NR2-receptor mRNA expression in the early stages of ischemia are observed prior to morphological evidence of neuronal damage or appearance of autoantibodies to them in blood serum specimens.

Thus, in one aspect the present invention provides a method for diagnosing a central nervous system disorder comprising measuring the level of NR2A and/or NR2B NMDA receptor or fragment thereof in a biological sample. Elevated levels of NR2A and NR2B NMDA receptors are specific to brain injury, and are expressed in ischemic brain tissue at higher rates than other NMDA receptors, and thus are uniquely suited for assessing ischemic brain episodes such as TIA or stroke. Baseline levels for determining whether the measured levels are elevated, and hence indicative of a central nervous system disorder, can be obtained from population norms or, preferably, from a patient's own test history.

The biological sample tested for the receptor or fragment can be derived from blood, urine, blood plasma, blood serum, cerebrospinal fluid, saliva, perspiration, or brain tissue. In a preferred embodiment, the biological sample is a blood sample. In an even more

preferred embodiment the biological sample is a blood sample diluted to a ratio of from about 1:2 to about 1:32 (v:v).

Immunoassay techniques are generally preferred for measuring the proteins or peptides of the present invention, as discussed in greater detail herein, although other analytical techniques are also available as known to those skilled in the art, such as HPLC. The amino acid sequences of the NR2A and NR2B subunits, and antigenic fragments thereof, are recited in SEQ ID NOS.1, 2, 3, 10, 11, and 12, and any fragment of these sequences can be employed in methods for directly detecting the receptors as long as sufficient antigenicity is maintained. However, when using immunoassays it has been found that the antigenic determinants are concentrated in the N-terminal domain of the NR2A and/or NR2B NMDA receptor, and that antibodies raised against the N-terminal domains and fragments thereof should be employed for optimal test results. The inventors have sequenced the amino acid chain of the N-terminal domains for these receptors, and set forth the sequences as SEQ ID NOS. 2 and 11, respectively, at the end of this document.

In a preferred embodiment, other biomarkers of central nervous system disorders are also measured to improve the accuracy of the diagnosis, and to provide further information about the nature, severity, or progression of the disorder. Particularly useful markers are directly implicated in the NMDA receptor pathway, and include naturally occurring agonists and antagonists of the NMDA receptors. An exemplary antagonist is glycine. Exemplary agonists include glutamate, polyglutamate, aspartate, polyaspartate, homocysteine, and polyhomocysteine. A particularly preferred agonist for measuring the activity of the receptors is glutamate or polyglutamate.

In another embodiment, thromboembolic biomarkers are measured to obtain a simultaneous reading of the likelihood for clotting in the brain. Exemplary thromboembolic biomarkers include homocysteine or polyhomocysteine.

Titers of higher than 2.63 for combined levels of NR2A and NR2B, especially when combined with titers higher than 3.34 for glutamate and/or 2.23 for homocysteine, are remarkably predictive of the occurrence of stroke and typically justify immediate therapeutic intervention for the TIA or stroke or risk of stroke. These titers can be translated into absolute concentrations by reference to the examples hereof.

The methods of the present invention are preferably performed by directly measuring the levels of NR2A and/or NR2B biomarkers in a selected biological sample, using immunoassay techniques employing antibodies raised against the biomarkers, or

Latex Agglutination and Other Diagnostic Techniques

Latex agglutination assays have been described in Beltz, G. A. et al., in *Molecular Probes: Techniques and Medical Applications*, A. Albertini et al., eds., Raven Press, New York, 1989, incorporated herein by reference. In the latex agglutination assay, antibody raised against a particular biomarker is immobilized on latex particles. A drop of the latex particles is added to an appropriate dilution of the serum to be tested and mixed by gentle rocking of the card. With samples lacking sufficient levels of the biomarkers, the latex particles remain in suspension and retain a smooth, milky appearance. However, if biomarkers reactive with the antibody are present, the latex particles clump into visibly detectable aggregates.

The term latex agglutination is employed generically herein to refer to any method based upon the formation of detectable agglutination, and is not limited to the use of latex as

the immunosorbent substrate. While preferred substrates for the agglutination are latex based, such as polystyrene and polypropylene, particularly polystyrene, other well-known substrates include beads formed from glass, paper, dextran, and nylon. The immobilized antibodies may be covalently, ionically, or physically bound to the solid-phase immunoabsorbent, by techniques such as covalent bonding via an amide or ester linkage, ionic attraction, or by adsorption. Those skilled in the art will know many other suitable carriers for binding antibodies, or will be able to ascertain such, using routine experimentation.

Thus, in one embodiment, the method of measuring the NR2A and/or NR2B NMDA receptor, fragment thereof, or other biomarker is by latex agglutination comprising:

(i) contacting the biological sample with poly- or monoclonal antibodies bound on an agglutinating carrier to target biomarkers for a sufficient time period and under conditions to promote agglutination; and

(ii) reading a signal generated from the agglutination; wherein the amount of signal detected correlates to the titer of biomarkers present in the sample.

The reaction is preferably read macroscopically against a dark background for a sufficient time period. The method preferably yields a clinically useful reading within about 30 minutes or less.

It has been experimentally found that latex beads having a mean diameter of from about 0.25 to about 0.4 μm are particularly preferred in the practice of this invention. The poly- or monoclonal antibodies are preferably present in a ratio with the latex beads of about 1:1.

Latex beads having the foregoing characteristics can be prepared generally by adding antibodies to the target biomarker to a carrier solution that contains a 1% concentration (by weight) of latex beads, until the concentration of the antibodies in the carrier solution reaches about 2 mg/ml, and allowing the ingredients a sufficient time to covalently link, typically about 1 hour, in the presence of a linking agent such as glutaraldehyde.

Conventional methods can be used to prepare the antibodies. For example, by using a peptide of a NMDA protein, polyclonal antisera or monoclonal antibodies can be made using standard methods. A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide (preferably the NR2A and/or NR2B receptor, an antigenic determinant of the NR2A and/or NR2B receptor, or an analogue or derivative

thereof) which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be administered and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g., the hybridoma technique originally developed by Kohler and Milstein (Nature 256, 495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., Immunol. Today 4, 72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. Monoclonal Antibodies in Cancer Therapy (1985) Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., Science 246, 1275 (1989)). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated. Therefore, the invention also contemplates hybridoma cells secreting monoclonal antibodies with specificity for NR2A or NR2B NMDA proteins or fragments thereof as described herein.

When the NR2A and/or NR2B receptors are detected indirectly, by measuring the cDNA expression of the NR2A and/or NR2B receptors, the measuring step in the present invention may be carried out by traditional PCR assays such as cDNA hybridization, Northern blots, or Southern blots. These methods can be carried out using oligonucleotides encoding the polypeptide antigens of the invention. Therefore, in one embodiment the methods are performed employing oligonucleotides that encode the amino acid sequence of SEQ ID NO: 2, which is preferably represented by nucleotides 371-1978 of SEQ ID NO: 6. More preferably, the nucleic acid construct comprises a oligonucleotide consisting of a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 3, which is preferably represented by oligonucleotides 1790-1852 of SEQ ID NO: 7.

Thus, in one embodiment the methods of this invention include measuring an increase of NR2A and/or NR2B cDNA expression by contacting the total DNA isolated from a biological sample with oligonucleotide primers attached to a solid phase, for a

sufficient time period. In another preferred embodiment, NR2A and/or NR2B cDNA expression is measured by contacting an array of total DNA bound to a solid matrix with a ready-to-use reagent mixture containing oligonucleotide primers for a sufficient time period. Expressed NR2A cDNA is revealed by the complexation of the cDNA with an indicator reagent that comprises a counterpart oligonucleotide to the cDNA attached to a signal-generating compound. The signal-generating compound is preferably selected from the group consisting of horseradish peroxidase, alkaline phosphatase, urinase and non-enzyme reagents. The signal-generating compound is most preferably a non-enzyme reagent.

In a preferred embodiment, the solid phase is a polymer matrix. More preferably, the polymer matrix is polyacrylate, polystyrene, or polypropylene. In one preferred embodiment the solid phase is a microplate. In another preferred embodiment, the solid phase is a nitrocellulose membrane or a charged nylon membrane.

As mentioned above, the methods of performing the present invention also may be performed by measuring the levels of autoantibodies specific for the NR2A and/or NR2B subunits. These autoantibodies may be measured by any suitable immunoassay such as, for example, a radioimmunoassay, an immunofluorescence assay, an enzyme-linked immunosorbent assay (ELISA), an immunocytochemical assay, and immunoblotting. In a preferred embodiment, the antigen to which the anti-NR2A and/or NR2B autoantibodies bind is a polypeptide or protein fragment of the N-terminal domain of the NR2A and/or NR2B receptor. More preferably, the antigen comprises a polypeptide or protein fragment of amino acid SEQ ID NO:2, 3, 4, 11, 12, or 13, or a suitable analogue or derivative thereof.

Thus, in yet another embodiment the methods of the present invention are performed by measuring the levels of anti-NR2A and/or anti-NR2B autoantibodies, by contacting a biological sample with a polypeptide or protein fragment of the NR2A and/or NR2B receptor (preferably the N-terminal domain) (or an analogue or derivative thereof) attached to a solid phase, for a sufficient time period and under conditions to allow a complex to form between any NR2A and/or NR2B autoantibodies which may be present in the sample and the polypeptide or protein fragment, contacting the complex with an indicator reagent comprising a secondary antibody specific for the species of the mammal attached to a signal-generating compound (or for the polypeptide or protein fragment); and measuring the signal generated. The peptide can be obtained directly from biological samples, by recombinant DNA techniques, or by direct chemical synthesis. The signal-generating compound is preferably selected from horseradish peroxidase, alkaline phosphatase, and

urinase. More preferably, the signal-generating compound is horseradish peroxidase. Most preferably, the indicator reagent is rabbit anti-human IgG attached to horseradish peroxidase. The amount of signal detected is correlated to the amount of anti-NR2A and/or NR2B autoantibodies present in the biological sample.

In this method it is preferred that the solid phase be a polymer matrix. More preferably, the polymer matrix is selected from the group consisting of polyacrylate, polystyrene, and polypropylene. In one preferred embodiment the solid phase is a microplate. In another preferred embodiment, the solid phase is a nitrocellulose membrane or a charged nylon membrane.

The immunosorbent of the present invention for measuring levels of autoantibody can be produced as follows. A fragment of the receptor protein is fixed, preferably by covalent bond or an ionic bond, on a suitable carrier such as polystyrene or nitrocellulose. If the standard polystyrene plate for immunological examinations is employed, it is first subjected to the nitration procedure, whereby free nitrogroups are formed on the plate surface, which are reduced to amino groups and activated with glutaric dialdehyde serving as a linker. Next the thus-activated plate is incubated with about 2 to 50 nM of the target peptide for the purpose of chemically fixing the respective immunogenic fragment of the receptor protein for a time and at a temperature sufficient to assure fixation (i.e. for about 16 hours at 4°C).

The amount of protein below 2 nM affects adversely the reliability of the findings, whereas its amount exceeding 50 nM is inexpedient due to an increase in the nonspecific binding of autoantibodies with the immunosorbents. The plate is then washed with an aqueous solution of sodium boron hydride and an aqueous solution of sodium chloride, vacuum-dried, enclosed in a hermetically sealed package, and put under storage at 4°C.

It is also practicable to produce the immunosorbent by fixing the respective fragment of the receptor protein on nitrocellulose strips by virtue of ionic interaction. The respective fragment of the receptor protein isolated from the mammals' brain is applied to nitrocellulose and incubated for 15 min at 37°C. Then nitrocellulose is washed with a 0.5 % solution of Tween-20, and the resultant immunosorbent is dried at room temperature and stored in dry place for one year period.

Emergency Room Diagnosis and Prognosis

As mentioned above, the methods of the present invention are especially well suited for use in emergency room settings. There are two reasons for this. First, the method is extremely useful in an emergency room setting because NR2A and NR2B NMDA receptor levels are elevated at a very early stage of ischemic insult, and thus provide a real time indication of neurotoxic events. This is in contrast to autoantibodies which require that an immune response first be mounted by the insulted organism.

The second reason the method is useful in an emergency room setting is the speed and ease with which the latex agglutination procedure can be employed. Using the latex agglutination processes described herein, one is able to turn laboratory results around often in less than 30 or even 20 minutes. Thus, using the methods of the present invention real-time data can be obtained that permits a therapeutic response within the window for an effective response to stroke.

Therefore, in one embodiment the invention provides a method for diagnosing the existence of a central nervous system disorder such as TIA or stroke, further comprising withdrawing the biological sample from a human subject, wherein the biological sample is withdrawn within three hours of the onset of symptoms of the central nervous system disorder. In still another embodiment of the invention, the amount of time elapsed between withdrawing the biological sample from the subject, and detecting or measuring the presence or quantity of the NR2A and/or NR2B NMDA receptor, is less than about one hour, 45 minutes, or 30 minutes.

One of the principal advantages of the present invention is the ability to distinguish ischemic episodes such as stroke from other brain injuries such as traumatic brain injury. Thus, in another embodiment, the invention provides a method for diagnosing the existence of TIA or stroke further comprising evaluating from the level of NR2A and/or NR2B NMDA receptor whether the brain injury is a traumatic brain injury or stroke/TIA, and administering traumatic brain injury or stroke/TIA therapy as appropriate.

Another advantage of the methods of the present invention which is extremely useful in an emergency room setting, is the ability to determine from the test data the type of stroke involved. In particular, if a stroke is suspected, the method will help diagnose whether the stroke is an ischemic or hemorrhagic insult. Thus, in another embodiment the invention provides a method for diagnosing the existence of TIA or stroke further comprising, when the diagnosis confirms a stroke, evaluating from the level of NR2A

and/or NR2B NMDA receptor whether the stroke is ischemic or hemorrhagic and administering ischemic or hemorrhagic stroke therapy as appropriate.

Another advantage of the present invention is the ability to evaluate infarction volume and extent of neurotoxicity from NMDA expression data. NMDA receptor expression research in an animal model of middle carotid artery occlusion has been employed to demonstrate such correlation. Thus, in still another embodiment the invention provides a method for diagnosing the existence of TIA or stroke further comprising, if TIA and/or stroke is confirmed, evaluating from the level of NR2A and/or NR2B NMDA receptor cranial infarct volume, and administering therapy appropriate to the infarct volume.

Moreover, one can periodically repeat the procedure, to provide continuous monitoring of a patient's state as interventional therapy is administered, to monitor the effectiveness of a particular therapeutic regime. In this embodiment, it is preferable for the mammal to be concurrently undergoing treatment for the disorder. More preferably, the samples are collected at intervals from about 20 min to about 1 month. Even more preferably, the interval is from about 20 min. to about 2 hours. Most preferably the samples are collected at an interval of about 30 minutes. Thus, in still another embodiment the invention provides a method for diagnosing the progression of TIA or stroke further comprising detecting or measuring the presence or quantity of a NR2A and/or NR2B NMDA receptor in a biological sample one or more additional times, at a frequency of less than about 6 hours.

Primary Care Physician Setting

In another application the method is used in a clinical setting to determine an individual's risk of stroke, or to monitor the effectiveness of risk reduction therapies. As mentioned above, a number of therapies can be employed to reduce the risk of stroke in an individual. The use of antiplatelet agents, particularly aspirin, is a standard treatment for patients at risk for stroke. People with atrial fibrillation (irregular beating of the heart) may be prescribed anticoagulants. The most important treatable factors linked to TIAs and stroke are high blood pressure, cigarette smoking, heart disease, carotid artery disease, diabetes, and heavy use of alcohol. Medical help is available to reduce and eliminate these factors. Lifestyle changes such as eating a balanced diet, maintaining healthy weight, exercising, and enrolling in smoking and alcohol cessation programs can also reduce these factors.

When these therapies are administered it is desirable to determine the effectiveness of the therapy.

Therefore, in one embodiment the invention provides a method for evaluating an individual's risk for TIA or stroke comprising measuring levels of NR2A and/or NR2B NMDA receptors or fragments thereof in a biological sample from the individual, and comparing the levels to a baseline level. In one embodiment the baseline levels are derived from population averages. In another embodiment the baseline levels are derived from the individual's own medical history.

In another embodiment the method is performed more than once to monitor the reduction or increase in risk for stroke or TIA, optionally in conjunction with the administration of risk reduction therapy. In one embodiment the method is performed at a frequency of from about one week to about six months. In another embodiment the method is performed at a frequency of from about one month to about three months.

In a particularly preferred embodiment other biomarkers are also measured to assess the risk for stroke or TIA. Particularly preferred biomarkers for risk of stroke or TIA are glutamate and homocysteine.

Novel Kits of the Present Invention

In another embodiment the invention provides kits for diagnosing central nervous system disorders such as TIA, stroke, and traumatic brain injury. NR2A and/or NR2B antibodies or antigens may be incorporated into immunoassay diagnostic kits depending upon whether autoantibodies or NMDA receptors are being measured. A first container may include a composition comprising an antigen or antibody preparation. Both antibody and antigen preparations should preferably be provided in a suitable titrated form, with antigen concentrations and/or antibody titers given for easy reference in quantitative applications.

The kits may also include an immunodetection reagent or label for the detection of specific immunoreaction between the provided antigen and/or antibody, as the case may be, and the diagnostic sample. Suitable detection reagents are well known in the art as exemplified by radioactive, enzymatic or otherwise chromogenic ligands, which are typically employed in association with the antigen and/or antibody, or in association with a second antibody having specificity for first antibody. Thus, the reaction is detected or quantified by means of detecting or quantifying the label. Immunodetection reagents and

processes suitable for application in connection with the novel methods of the present invention are generally well known in the art.

The reagents may also include ancillary agents such as buffering agents and protein stabilizing agents, e.g., polysaccharides and the like. The diagnostic kit may further include where necessary agents for reducing background interference in a test, agents for increasing signal, apparatus for conducting a test, calibration curves and charts, standardization curves and charts, and the like.

In a more particular aspect the invention relates to a rapid multiple panel containing antibodies to the thromboembolic and neurotoxicity biomarkers glutamate, homocysteine and NMDA receptors that employs latex agglutination. Thus, in one embodiment the invention provides a kit for diagnosing central nervous system disorders comprising: (1) an agglutinating immunosorbent for NR2A and/or NR2B NMDA receptors or fragments thereof, and (2) a control such as saline or a known concentration of NR2A and/or NR2B receptor or fragment thereof. In a more preferred embodiment the kit further comprises an agglutinating immunosorbent for another biomarker for TIA/stroke, such as an agonist or antagonist of NR2A and/or NR2B, a thromboembolic marker, or more particularly glutamate or polyglutamate, and/or an agglutinating homocysteine or polyhomocysteine. The agglutinating immunosorbent is preferably of the type discussed in greater detail above.

In another embodiment the invention relates to a kit for detecting NR2A and/or NR2B receptors or fragments thereof that does not employ latex agglutination. Thus, in another embodiment the invention provides a kit for diagnosing central nervous system disorders comprising: (1) an immunosorbent for NR2A and/or NR2B NMDA receptors or fragments thereof, and (2) an indicator reagent comprising secondary antibodies attached to a signal generating compound. The secondary antibodies can be specific for the receptor or fragment, or for the primary antibodies in the immunosorbent. In a preferred embodiment the kits further comprise an immunosorbent for glutamate or polyglutamate, and/or an immunosorbent for homocysteine or polyhomocysteine, and secondary antibodies against the glutamate and/or homocysteine, or to the primary antibodies on the immunosorbents against the glutamate or homocysteine. The immunosorbent preferably comprises anti-antibodies for the biomarkers bound to a solid support.

In another aspect the present invention relates to a test-kit that relies upon PCR amplification for measuring NR2A and/or NR2B levels. Thus, in another embodiment the invention provides a kit comprising: (a) one or more oligonucleotide primers (preferably of

SEQ ID NO: 8) attached to a solid phase, (b) indicator reagent attached to a signal-generating compound capable of generating a detectable signal from oligonucleotides, and (c) a control sample (i.e. template cDNA). The reagents may also include ancillary agents such as buffering agents, polymerase agents, and the like. The diagnostic kit may further include, where necessary, other members of the signal-producing system of which system the detectable group is a member (e.g., enzyme and non-enzyme substrates), agents for reducing background interference in a test, agents for increasing the signal, apparatus for conducting a test, and the like.

In another embodiment of test-kit comprises (a) a solid phase to which biological fluids for receiving total DNA including NR2A cDNA could be attached, (b) oligonucleotide primers, preferably in a ready-to-use PCR buffer, and (c) a control sample (i.e. template cDNA). Ancillary agents as described above may similarly be included.

In another embodiment the invention provides a diagnostic kit for detecting NR2A and/or NR2B autoantibodies comprising (a) a polypeptide of the N-terminal domain of the NR2A and/or NR2B receptor, fragment thereof, or analog or derivative thereof, (b) an indicator reagent comprising a secondary antibody specific for the autoantibody or the polypeptide attached to a signal-generating compound; and (c) a control sample, such as a known concentration of NR2A and/or NR2B polyclonal antibodies. The reagents may also include ancillary agents such as buffering agents and protein stabilizing agents, e.g., polysaccharides and the like. The diagnostic kit may further include, where necessary, other members of the signal-producing system of which system the detectable group is a member (e.g., enzyme and non-enzyme substrates), agents for reducing background interference in a test, agents to increase the signal, apparatus for conducting a test, calibration and standardization information or instructions, and the like.

Novel Compositions of the Invention

The methods of the present invention rely upon a series of novel compositions which themselves form a part of the invention. Thus, in one series of embodiments the invention provides an isolated polypeptide fragment of the NR2A and/or NR2B NMDA receptor, comprising:

1. An antigenic determinant of the NR2A NMDA receptor,
2. An antigenic determinant of the NR2B NMDA receptor,
3. The N-terminal domain of the NR2A NMDA receptor,

4. The N-terminal domain of the NR2B NMDA receptor,
5. SEQ ID NO. 2,
6. SEQ ID NO. 3,
7. SEQ ID NO. 4,
8. SEQ ID NO. 11,
9. SEQ ID NO. 12, and
10. SEQ ID NO. 13,

or an antigenic fragment, analog, or derivative thereof. In another series of embodiments the invention provides any of the foregoing polypeptides linked covalently to a distinct antigenic determinant, such as human serum albumin. In still another series of embodiments the invention provides any of the foregoing polypeptides linked to any of the immunosorbent materials discussed above. The immunosorbent can be in the form of a bead for latex agglutination, in the size ranges discussed above, or in the form of a synthetic plate for conventional immunoassay analysis. The polypeptide can be linked to the immunosorbent using any conventional means of linkage, including covalent linkage, ionic linkage, and adsorption.

In another series of embodiments the present invention relates to the novel monoclonal and polyclonal antibodies specific for and/or raised against the foregoing polypeptides, including the foregoing polypeptides linked to distinct antigenic determinants. Thus, in one embodiment the invention provides non-human antibodies against any of the foregoing peptides or polypeptides or antigenic fragment, analog, or derivative thereof. In another embodiment the invention provides immunosorbents to which such antibodies are linked.

In another series of embodiments the present invention provides oligonucleotides that encode the foregoing peptides and polypeptides and fragments, analogs, and derivatives thereof, and to recombinant expression vectors that include such oligonucleotides. Such oligonucleotides include, without limitation, the oligonucleotides defined by SEQ ID NO:6, 7, 14, and 15, and fragments thereof which encode antigenic determinants.

In still another embodiment the present invention relates to isolated oligonucleotide sequences that are useful in the cDNA PCR analytical techniques of the present invention. Thus, the invention further provides oligonucleotides comprising the nucleotide sequences of SEQ ID NOS:7, 8, 9, 15, 16, and 17.

BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

The features, aspects, and advantages of the present invention will become better understood with regard to the following sequence listings where, in the sequence the recited amino acid position numbering reflects that used throughout this document.

SEQ ID NO:1. shows the full-length amino acid sequence of the mature NR2A receptor subunit, as follows:

SEQUENCE LISTING

PEPTIDE

Homo sapiens glutamate receptor. ionotropic, N-methyl D-aspartate 2A

Science 256:1217-1221(1992)

NCBI/NM 000833.2

1	11	21	31	41
51				
1				
MGR LGYWTLL VLPALLVWRD PAQNAAAEKG PPALNIAVLL GHSHDVTRE				
60				
61				
TGLPLDVNVV ALLMNRTDPK SLITHVCDLM SGARIHGLVF GDDTDQEAVA				
120				
121				
FIPILGIHGG ASMIMADKDP TSTFFQFGAS IQQQATVMLK IMQDYDWHVF				
180				
181				
RDFISFIKTT VDNSFVGWDM QNVITLDTSF EDAKTQVQLK KIHSSVILLY				
240				
241				
SEARSLGLTG YDFFWIVPSL VSGNTELPK EFPSGLISVS YDDWDYSLEA				
300				
301				
TAASSMLEKF SYIPEAKASC YGQAEKPETP LHTLHQFMVN VTWDGKDLSF				
360				
361				
LVVIVLNKDR EWEKVGKWEN QTLSLRHAVW PRYKSFS DCE PDDNHLSIVT				
420				
421				
DIDPLTETCV RNTVPCRKFV KINNSTNEGM NVKKCKKGFC IDILKKLSRT				
480				
481				
TNGKHGKKVN NVWNGMIGEV VYQRAVMAVG SLTINEERSE				
540				
VVDFSVPFVE TGISVMVSRS				

541 NGTVSPSAFL EPFSASVWVM MFVMLLIVSA IAVFVFEYFS PVGYNRNLAK
GKAPHGPSFT 600

601 IGKAIWLLWG LVFNNSVPVQ NPKGTTSKIM VSVWAFFAVI FLASYTANLA
AFMIQEEFVD 660

661 QVTGLSDKKF QRPHDYSPPF RFGTVPNGST ERNIRNNYPY MHQYMTRFNQ
RGVEDALVSL 720

721 KTGKLDAFIY DAAVLNYKAG RDEGCKLVTI GSGYIFASTG YGIALQKGSP
WKRQIDLALL 780

781 QFVGDGEMEE LETLWLTGIC HNEKNEVMSS QLDIDNMAGV
FYMLAAAMAL SLITFIWEHL 840

841 FYWKLRFCFT GVCSDRPGLL FSISRGIYSC IHGVHIEKK KSPDFNLTGS
QSNMLKLLRS 900

901 AKNISNMSNM NSSRMDSPKR ATDFIQRGSL IVDMVSDKGN LIYSDNRSFQ
GKDSIFGDNM 960

961 NELQTFVANR HKDNLSNYVF QGQHPLTLNE SNPNTVEVAV STESKGNSRP
RQLWKKSMEs 1020

1021 LRQDSLQNQP VSQRDEKTAE NRTHSLKSPR YLPEEVAHSD ISETSSRATC
HREPDNNKNH 1080

1081 KTKDNFKRSM ASKYPKDCSD VDRTYMKTKA SSPRDKIYTI DGEKEPSFHL
DPPQFVENIT 1140

1141 LPENVGFPDT YQDHNEFRK GDSTLPMNRN PLHNEDGLPN
NDQYKLYAKH FTLKDKGSPH 1200

1201 SEGSDRYRQN STHCRSCLSN LPTYSGHFTM RSPFKDACL RMGNLYDIDE
DQMLQETGNP 1260

1261 ATREEVYQQD WSQNNALQFQ KNKLRINRQH SYDNILDKPR EIDLSRPSRS
ISLKDRERLL 1320

1321 EGNLYGSLFS VPSSKLLGNK SSLFPQGLED SKRSKSLLPD HASDNPFLHT
YGDDQRLVIG 1380

1381 RCPSPYKHS LPSQAVNDSY LRSSLRSTAS YCSRDSRGHS DVYISEHVMP
YAANKNTMYS 1440

1441 TPRVLNCSN RRVYKKMPSI ESDV

SEQ ID NO:2. shows the amino acid sequence of the auto-antigenic region of the N-terminal domain of the NR2A subunit, as follows:

SEQ ID NO:2

HOMO SAPIENS

PAQNAAAEKG PPALNIAVLL GHSHDVTERE LRNLWGPEQA 60
 61 TGLPLDVNVV ALLMNRTDPK SLITHVCDLM SGARIHGLVF GDDTDQEAVA
 QMLDFISSQT 120
 121 FIPILGIHGG ASMIMADKDP TSTFFQFGAS IQQQATVMLK IMQDYDWHVF
 SLVTTIFPGY 180
 181 RDFISFIKTT VDNSFVGWDM QNVITLDTSF EDAKTQVQLK KIHSSVILLY
 CSKDEAVLIL 240
 241 SEARSLGLTG YDFFWIVPSL VSGNTELIPK EFPSGLISVS YDDWDYSLEA
 RVRDGLGILT 300
 301 TAASSMLEKF SYIPEAKASC YGQAEKPETP LHTLHQFMVN VTWDGKDLSF
 TEEGYQVHPR 360
 361 LVVIVLNKDR EWEKVGKWEN QTLRLHAVW PRYKSFSDCE PDDNHLSIVT
 LEEAPFVIVE 420
 421 DIDPLTETCV RNTVPCRKFV KINNSTNEGM NVKKCKKGFC IDILKKLSRT
 VKFTYDLYLV 480
 481 TNGKHGKKVN NVWNGMIGEV VYQRAVMAVG SLTINEERSE
 VVDFSVPFVE TGISVMVSRS 540
 541 NGTVSPSAFL EPFSAS

SEQ ID NO:3; shows a 21 amino acid antigenic peptide, corresponding to a fragment of the NR2A N-terminal domain. another such peptide (21 amino acids derived from the NR2A sequence and an N-terminal Cys for attachment to a carrier protein), as follows:

SEQ ID NO:3

Homo sapiens

NGMIGEVVYQRAVMAVGSLTI

092021-0804-0000

SEQ ID NO:4. shows a 22 amino acid antigenic peptide, corresponding to a fragment of the NR2A N-terminal domain.another such peptide, modified by an N-terminal Cys for attachment to a carrier protein):

Artificial Sequence

CNGMIGEVVYQRAVMAVGSLTI

FULL

BASE COUNT ORIGIN

Homo sapiens glutamate receptor, ionotropic, N-methyl D-aspartate 2A (GRIN 2A) mRNA

SEQ ID NO:5. shows the Oligonucleotide position numbering used throughout in reference to NR2A oligonucleotide sequences, as follows:

SEQ ID NO:5

Science 256:1217-1221(1992)

May 22, 1992

NIGB/NM_000833

1 atcatgggac cgggtgagcg ctgagaatcg cggccgcagc catcagccct ggagatgacc
 61 aggagcggcc actgctgaga actatgtgga gagaggctgc gagccctgct gcagagcctc
 121 cggctgggat agccgcccc cgtgggggcg atgcggacag cgcgggacag ccaggggagc
 181 gcgctggggc cgcagcatgc gggaaccgc taaaccggg ggctgctgag gcggccgaga
 241 tgctgtgctg cgcagcgcgc cccactgcat cctcgacctt ctcgggctac agggaccgtc
 301 agtggcgact atgggcagag tgggctattg gacctgtctg gtgctgccgg cccttctggt
 361 ctggcgcggt ccggcgccga gcgcggcggc ggagaagggt ccccccgcgc taaatattgc
 421 ggtgatgctg ggtcacagcc acgacgtgac agagcgcgaa cttcgaacac tgtggggccc
 481 cgagcaggcg gcggggctgc ccttgacgt gaacgtggtg gctctgctga tgaaccgcac
 541 cgacccaag agcctcatca cgcacgtgtg cgacctcatg tccggggcac gcatccacgg
 601 cctcgtgttt ggggacgaca cggaccagga ggccgtagcc cagatgctgg attttatctc
 661 ctccacacc ttctcccca tcttgggcat tcatgggggc gcatctatga tcatggctga
 721 caagatccg acgtctacct tcttcagtt tggagcgtcc atccagcagc aagccacggt
 781 catgctgaag atcatgcagg attatgactg gcatgtcttc tccctggtga ccactatctt
 841 ccctggctac aggggaattca tcagcttctg caagaccaca gtggacaaca gctttgtggg
 901 ctgggacatg cagaatgtga tcacttgga cacttcctt gaggatgcaa agacacaagt
 961 ccagctgaag aagatccact cttctgtcat cttgctctac tgttccaaag acgaggctgt

1021 tctcattctg agtgaggccc gctcccttgg cctcaccggg tatgatttct tctggattgt
 1081 cccagcttg gtctctggga acacggagct catcccaaaa gagtttccat cgggactcat
 1141 ttctgtctcc tacgatgact gggactacag cctggaggcg agagtgaggg acggcattgg
 1201 catcetaacc accgctgcat cttctatgct ggagaagttc tcctacatcc ccgaggccaa
 1261 ggccagctgc tacgggcaga tggagaggcc agaggtcccg atgcacacct tgcaccatt
 1321 tatggtcaat gttacatggg atggcaaaga cttatccttc actgaggaag gctaccaggt
 1381 gcacccagg ctggtgtga ttgtgtgaa caaagaccgg gaatgggaaa aggtgggcaa
 1441 gtgggagaac catacgtga gcctgaggca cgccgtgtgg ccaggtaca agtccttctc
 1501 cgactgtgag ccggtatgaca accatctcag catcgtcacc ctggaggagg cccattcgt
 1561 catcgtggaa gacatagacc ccctgaccga gacgtgtgtg aggaacaccg tgccatgtcg
 1621 gaagtctgc aaaatcaaca attcaacaa tgaggggatg aatgtgaaga aatgctgcaa
 1681 ggggttctgc attgatattc tgaagaagct ttccagaact gtgaagtta cttacacct
 1741 ctatctgggtg accaatggga agcatggcaa gaaagttaac aatgtgtgga atggaatgat
 1801 cggtgaagtg gtctatcaac gggcagtcac ggcagttggc tcgctacca tcaatgagga
 1861 acgttctgaa gtggtggact tctctgtgcc ctttgtggaa acgggaatca gtgtcatggt
 1921 ttcaagaagt aatggcaccg tctcaccttc tgcttttcta gaaccattca gcgcctctgt
 1981 ctgggtgatg atgttttga tgctgtcat tgtttctgcc atagctgttt ttgtcttga
 2041 atacttcagc cctgttgat acaacagaaa cttagccaaa gggaaagcac cccatgggcc
 2101 ttcttttaca attgaaaag ctatatggct tctttggggc ctggtgttca ataactcgt
 2161 gcctgtccag aatcctaaag ggaccaccag caagatcatg gtatctgtat gggccttctt
 2221 cgctgtcata ttctggcta gctacacagc caatctggct gccttcatga tccaagagga
 2281 atttgtggac caagtaccg gcctcagtga caaaaagttt cagagacctc atgactattc
 2341 cccacctttt cgatttggga cagtgcctaa tggaagcacg gagagaaaca ttcggaataa
 2401 ctatccctac atgcatcagt acatgaccaa atttaacag aaaggagtag aggacgcctt
 2461 ggtcagcctg aaaacgggga agctggacgc ttcatctac gatgccgcag tcttgaatta
 2521 caaggctggg agggatgaag gctgcaagct ggtgaccatc gggagtgggt acatctttgc
 2581 caccaccggt tatggaattg ccttcagaa aggtctcct tggaagaggc agatcgacct
 2641 ggccttgctt cagtttgtgg gtgatgtga gatggaggag ctggagacct tgtggctcac
 2701 tgggatctgc cacaacgaga agaacgaggt gatgagcagc cagctggaca ttgacaacat
 2761 ggcgggcgta ttctacatgc tggctgccgc catggccctt agcctcatca ccttcatctg
 2821 ggagcacctc ttctactgga agctgcgctt ctgtttcacg ggcgtgtgct ccgaccggcc
 2881 tgggttgctc ttctcatca gcaggggcat ctacagctgc attcatggag tgcacattga
 2941 agaaaagaag aagtctccag acttcaatct gacgggatcc cagagcaaca tgttaaaact

3001 cctccggtea gccaaaaaca ttccagcat gtccaacatg aactcctcaa gaatggactc
 3061 acccaaaaga gctgctgact tcatccaaag aggttcctc atcatggaca tggtttcaga
 3121 taaggggaat ttgatgtact cagacaacag gtcctttcag gggaaagaga gcatttttg
 3181 agacaacatg aacgaactcc aaacatttgt ggccaaccgg cagaaggata acctcaataa
 3241 ctatgtattc cagggacaac atcctcttac tctcaatgag tccaacccta acacggtgga
 3301 ggtggccgtg agcacagaat ccaaagcgaa ctctagacc cggcagctgt ggaagaaatc
 3361 cgtggattcc atacgccagg attcactatc ccagaatcca gtctcccaga gggatgaggc
 3421 aacagcagag aataggacc actccctaaa gagccctagg tatcttcag aagagatggc
 3481 ccactctgac atttcagaaa cgtcaaatcg ggccacgtgc cacagggaac ctgacaacag
 3541 taagaaccac aaaaccaagg acaacttta aaggtcagt gcctccaaat accccaagga
 3601 ctgtagttag gtcgagcgca cctacctgaa aaccaaata agctccccta gagacaagat
 3661 ctactata gatggtgaga aggagcctgg ttccactta gatccacccc agtttgtga
 3721 aaatgtgacc ctgcccaga acgtggactt cccggacccc taccaggatc ccagtgaata
 3781 ctccgcaag ggggactcca cgctgccaat gaaccggaac ccttgcata atgaagaggg
 3841 gctttccaac aacgaccagt ataaactcta ctccaagcac ttacattga aagacaaggg
 3901 ttccccgcac agtgagacca gcgagcgata ccggcagaac tccacgact gcagaagctg
 3961 ctttccaac atgccacct attcaggcca cttaccatg aggtcccct tcaagtgcga
 4021 tgcctgcctg cggatgggga acctctatga catcgatga gaccagatgc ttcaggagac
 4081 agtaacca gccaccggg agcaggctta ccagcaggac tgggcacaga acaatgccct
 4141 tcaattaca aagaacaagc taaggattag ccgtcagcat tctacgata acattgtga
 4201 caaacctagg gagctagacc ttgacaggcc ctccggagc ataagcctca aggacagga
 4261 acggcttctg gagggaaatt ttacggcag cctgtttagt gtcccctcaa gcaaactctc
 4321 ggggaaaaaa agctccctt tccccagg tctggaggac agcaagagga gcaagtctct
 4381 cttgccagac cacactccg ataaccctt cctccactcc cacagggatg accaacgctt
 4441 ggttattggg agatgccct cggacctta caaactctg ttgcatccc aggcggtgaa
 4501 tgacagctat ctccggtct ccttgaggtc aacggcatcg tactgttcca gggacagtcg
 4561 gggccacaat gatgtgtata ttccggagca tgttatgcct tatgtgcaa ataagaataa
 4621 tatgtactct acccccaggg tttaaattc ctgcagcaat agacgcgtgt acaagaaaat
 4681 gcctagtatc gaatctgatg tttaaaaatc ttccattaat gtttatcta tagggaaata
 4741 cacgtaatgg ccaatgttct ggagggtaaa tgttgatgt ccaatagtgc cctgctaaga
 4801 ggaagaagat gtagggaggt atttgtgtg tgtgtgtgt ggctctttg cacacggctt
 4861 catgccataa tctccactc aaggaatct gtgagggtgt tgctgagcat ggcagacacc
 4921 agataggatg gtccttaacc aaaaataact aactacataa gggcaagtct ccgggacatg

4981 cctactgggt atgttgcaa taatgatgca ttggatgcca atggtgatgt tatgattcc
 5041 tatattccaa attccattaa ggtcagccca ccatgtaatt ttctcatcag aaatgcctaa
 5101 tggtttctct aatacagaat aagcaatatg gtgtgcatgt aaacctgaca cagacaaaat
 5161 aaaaacagtt aagaatgcat ctgcactgta gtcggatttg aacatgtgca agagattagg
 5221 aagtttggct cgtaacagtt tcagctttct tgttatgcct tccatcacag cccaggctca
 5281 ccccaagaac tccaggctcc cctaaagaat agcaaatcag tgtgttcgtg atgactgtgc
 5341 taccttcatt atagttcatt tccaagacac atctggagcc aaaggcccg gggaccctca
 5401 ggtggggaga gctacaggaa tctctttgga tgttgatgtg tgtttctctc taccctcggc
 5461 ttcatggtc ttgttcagag ctgcataaac taacacattt atgtctccga gatctaagtg
 5521 tggatcttct gtctgtgaca cagtggccat ttagtttat cccgaagacg cctatgtacg
 5581 taagtttgca ttctctcct tctggtgatg actcagggtt gtatagtatc tgttaccct
 5641 tccctcccag agtaaccata actcgttccg ttccaaaca gccatggtgg tgtccaatta
 5701 gctgtgtatc gctcttcca gagttgttaa tgtggtgaca tgcaccaaca gccgtatgtg
 5761 tactgtgatc tgtaagaagt acaatgccat ctgtctgccg aaggctagca tggttttagg
 5821 tttatcttc ttcacatcca gaaattctgt tggacactca ctccacccc aaactctca
 5881 aatcaaaagc ctcaaaaca cgaggcactc ttggatctac cctgagtatc ctcaaactg
 5941 tggatacagt ttagtgagac aagcaattc tcccttctga gttattctct ctgttggtgg
 6001 caaaccactt catagcacca acagagatgt aggaaaaatt cctcaaagta ttgtcattt
 6061 ctgagtcgcc tgcattatcc cattettatt ctctcaaac ctgtgcatat atgacatgaa
 6121 atgatatcca tttttttt aagttagaaa cagagagggg aatacttatg catggggagc
 6181 ctgttagcac agtgcctgcc acaaaaacaa gtgccccga caagatagtt gctatgttat
 6241 gacactttct cagatcagga tttctagtt taaaaattaa atatcataaa acg

SEQ ID NO:6. shows the oligonucleotide sequence of the auto-antigenic region of the N-terminal domain of the NR2A subunit, as follows:

SEQ ID NO:6

N-terminal nucleotide sequence

371 ccggcgccga gcgcggcggc ggagaagggt cccccgcgc taaatattgc
 421 ggtgatgctg ggtcacagcc acgacgtgac agagcgcgaa ctccgaacac tgtggggccc
 481 cgagcaggcg gcggggctgc ccttgacgt gaacgtggta gctctgctga tgaaccgcac
 541 cgacccaag agcctcatca cgcacgtgtg cgacctcatg tccggggcac gcatccacgg
 601 cctcgtgttt ggggacgaca cggaccagga ggccgtagcc cagatgctgg attttatctc
 661 ctccacacc ttctccca tcttgggcat tcatgggggc gcattatga tcatggctga

721 caaggatccg acgtctacct tcttccagtt tggagcgtcc atccagcagc aagccacggt
 781 catgctgaag atcatgcagg attatgactg gcatgtcttc tccctggtga ccactatctt
 841 ccttggttac aggaattca tcagcttcgt caagaccaca gtggacaaca gctttgtggg
 901 ctgggacatg cagaatgtga tcacactgga cacttccttt gaggatgcaa agacacaagt
 961 ccagctgaag aagatccact cttctgtcat ctgtctctac tgttcaaag acgaggctgt
 1021 tctcattctg agtgaggccc gctcccttgg cctcaccggg tatgatttct tctggattgt
 1081 cccagcttg gtctctggga acacggagct catcccaaaa gagtttccat cgggactcat
 1141 ttctgtctcc tacgatgact gggactacag cctggaggcg agagtggagg acggcattgg
 1201 catctaacc accgctgcat cttctatgct ggagaagttc tctacatcc ccgaggccaa
 1261 ggccagctgc tacgggcaga tggagaggcc agaggcccc atgcacacct tgcacccatt
 1321 tatggtcaat gttacatggg atggcaaaga cttatccttc actgaggaag gctaccaggt
 1381 gcaccccagg ctggtggtga ttgtgctgaa caaagaccgg gaatgggaaa aggtgggcaa
 1441 gtgggagaac catacgtga gcctgaggca cgccgtgtgg cccaggtaca agtccttctc
 1501 cgactgtgag ccggtatgaca accatctcag catcgtcacc ctggaggagg cccattctgt
 1561 catcgtggaa gacatagacc ccctgaccga gacgtgtgtg aggaacaccg tgccatgtcg
 1621 gaagttcgtc aaaatcaaca attcaaccaa tgaggggatg aatgtgaaga aatgctgcaa
 1681 ggggttctgc attgatattc tgaagaagct ttccagaact gtgaagttta cttacgacct
 1741 ctatctggtg accaatggga agcatggcaa gaaagttaac aatgtgtgga atggaatgat
 1801 cgggtgaagt gtctatcaac gggcagtcac ggcagttggc tcgtcacca tc aatgagga
 1861 acgttctgaa gtggtggact tctctgtgcc ctttgtggaa acgggaatca gtgtcatggt
 1921 ttcaagaagt aatggcaccg tctcaccttc tgcttttcta gaaccattca gcgcctct

SEQ ID NO:7 shows a 62 oligonucleotide fragment target, as follows:

SEQ ID NO:7

atggaatgatcggatgaagtgtctatcaacgggcagtcagtcagttggctcgtcaccatc

SEQ ID NO:8 shows one oligonucleotide primer, as follows:

SEQ ID NO:8

agcatggcaagaagttaaca

SEQ ID NO:9 shows a second oligonucleotide primer, as follows:

SEQ ID NO:9

acgttctgaagtgggtggactt

SEQ ID NO:10. shows the full-length amino acid sequence of the mature NR2B receptor subunit, as follows:

PEPTIDE

Homo sapiens glutamate receptor, ionotropic, N-methyl D-aspartate 2B
Biochim. Biophys. Acta 1260:105-108(1995).

sequence NME2_HUMAN (Q13224)

1	11	21	31	41	51
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1 MKPRAECCSP KFWLVLA VLA VSGSRARSQK SPPSIGIAVI LVGTSDEVAI
KDAHEKDDFH 60
61 HLSVVRVEL VAMNETDPKS IITRICDLMS DRKIQGVVFA DDTDQEAIQA
ILDFISAQTL 120
121 TPILGIHGGG SMIMADKDES SMFFQFGPSI EQQASVMLNI MEEYDWYIFS
IVTTYFPGYQ 180
181 DFNKIRSTI ENSFVGWELE EVLLLDMSLD DGDSKIQNQL KKLQSPIILL
YCTKEEATYI 240
241 FEVANSVGLT GYGYTWIVPS LVAGD TDTP AEFPTGLISV SYDEWDYGLP
ARVRDGIAII 300
301 TTAASDMLSE HSFIEPKSS CYNTHKRIY QSNMLNRYLI NVTFEGRNLS
FSEDGYQMHP 360
361 KLVIILLNKE RKWERVGKWK DKSLQMKYYV WPRMCPETEE QEDDHLSIVT
LEEAPFVIVE 420
421 SVDPLSGTCM RNTVPCQKRI VTENKTDEEP GYIKKCKGFCIDILKKISK
SVKFTYDLYL 480
481 VTNGKHGKKI NGTWNGMIGE VVMKRAYMAV GSLTINEERS EVVDFSVPFI
ETGISVMVSR 540
541 SNGTVSPSAF LEPFSADVWV MMFVMLLIVS AVAVFVFEYF SPVGYNRCLA
DGREPGGPSF 600
601 TIGKAIWLLW GLVFNNVSPV QNPKGTTSKI MVSVWAFFAV IFLASYTANL
AAFMIQEEYV 660

SEQ ID NO:11. shows the amino acid sequence of the auto-antigenic region of the N-terminal domain of the NR2B subunit, as follows:

61 HLSVVPRVEL VAMNETDPKS IITRICDLMS DRKIQGVVFA DDTDQEAlAQ
ILDFISaQTL 120

121 TPILGIHGGG SMIMADKDES SMFFQFGPSI EQQASVMLNI MEEYDWYIFS
IVTTYFPGYQ 180

181 DFVNKIRSTI ENSFVGWELE EVLLLDMSLD DGDSKIQNQL KKLQSPIILL
YCTKEEATYI 240

241 FEVANSVGLT GYGYTWIVPS LVAGD TDTVP AEFPTGLISV SYDEWDYGLP
ARVRDGIAII 300

301 TTAASDMLSE HSFIEPKSS CYNTHKRIY QSNMLNRYLI NVTFEGRNLS
FSEDGYQMHP 360

361 KLVILLNKE RKWERVGKWK DKSLQMKYYV WPRMCPETEE QEDDHLISVT
LEEAPFVIVE 420

421 SVDPLSGTCM RNTVPCQKRI VTENKTDEEP GYIKKCCKGF CIDILKKISK
SVKFTYDLYL 480

481 VTNGKHGKKI NGTWNGMIGE VVMKRAYMAV GSLTINEERS EVVDFSVPFI
ETGISVMVSR 540

541 SNGTVSPSAF LEPFSAD

SEQ ID NO:12; shows a 20 amino acid antigenic peptide fragment of the NR2B subunit, as follows:

SEQ ID NO:12

Homo sapiens

GYIKKCCKGF CIDILKKISK

SEQ ID NO:13 shows a 21 amino acid sequence of an antigenic fragment of the NR2B subunit modified by an N-terminal Cys for attachment to a carrier protein, as follows:

SEQ ID NO:13.

Artificial Sequence (21 aminoacids)

CGYIKKCCKGF CIDILKKISK

FULL

BASE COUNT ORIGIN

SEQ ID NO:14 shows the oligonucleotide position numbering used throughout in reference to NR2B oligonucleotide sequences, as follows:

000001-000001

SEQ. NO. 14

Homo sapiens glutamate receptor, ionotropic, N-methyl D-aspartate 2B mRNA

1 ttgaatttgc atctcttcaa gacacaagat taaaacaaaa ttacgctaa attggatttt
61 aaattatctt ccgttcattt atccttcgtc ttcttatgt ggatatgcaa gcgagaagaa
121 gggactggac attccaaca tgctcactcc cttaatctgt ccgtctagag gtttggttc
181 taaaaaccaa gggagtcgac gaggtaaga tgaagcccag agcggagtcg tgtctccca
241 agttctgggt ggtgttggcc gtctggccg tgcaggcag cagagctctg tctcagaaga
301 gccccccag cattggcatt gctgtcatcc tcgtgggcac ttccgacgag gtggccatca
361 aggatgccc cagaaagat gattccacc atctctccgt ggtacccgg gtggaactgg
421 tagccatgaa tgagaccgac ccaaagagca tcatcaccg catctgtgat ctcattgtg
481 accggaagat ccaggggggtg gtgtttgctg atgacacaga ccaggaagcc atcggccaga
541 tctctgattt cattcagca cagactctca cccgatcct ggcatccac gggggctcct
601 ctatgataat ggcagataag gatgaatcct ccatgttctt ccagtttggc ccatcaattg
661 aacagcaagc ttccgaatg ctcaacatca tggaagaata tgactgttac atcttttcta
721 tcgtcaccac ctatttcct ggctaccagg actttgtaaa caagatccgc agcaccattg
781 agaatacctt tgtgggtggt gagctagagg aggtcctcct actggacatg tccctggacg
841 atggagattc taagatccag aatcagctca agaaactca aagccccatc attcttctt
901 actgtaccaa ggaagaagcc acctacatct ttgaagtggc caactcagta gggctgactg
961 gctatggcta cacgtggatc gtgccagtc tgggtggcagg ggatacagac acagtgcctg
1021 cggagttccc cactgggctc atctctgtat catatgatga atgggactat ggctccccg
1081 ccagagttag agatggaatt gccataatca ccactgctgc ttctgacatg ctgtctgagc
1141 acagcttcat ccctgagccc aaaagcagtt gttacaacac ccacgagaag agaattctacc
1201 agtccaatat gctaaatagg tatctgatca atgtcactt tgaggggagg aatttgcct
1261 tcaatgaaga tggctaccag atgcaccga aactggtgat aattcttctg aacaaggaga
1321 ggaagtggga aagggtgggg aagtggaaag acaagtcct gcagatgaag tactatgtgt
1381 ggccccgaat gtgtccagag actgaagagc aggaggatga ccatctgagc attgtgaccc
1441 tggaggaggc accatttgc attgtgaaa gtgtggacc tctgagtga acctgcatga
1501 ggaacacagt cccctgcaa aaacgcatag tcatgagaa taaaacagac gaggagccgg
1561 gttcatcaa aaaatgctgc aagggttct gtattgacat ccttaagaaa atttctaat
1621 ctgtgaagtt cacctatgac cttacctgg ttaccaatgg caagcatggg aagaaaatca
1681 atggaacctg gaatggtatg attggagagg tggcatgaa gagggcctac atggcagtgg
1741 gctcactcac catcaatgag gaacgatcg aggtggcga cttctctgtg ccttcatag
1801 agacaggcat cagtgtcatg gtgtcacga gcaatgggac tgtctacct tctgccttct

092301 08001
T022660

1861 tagagccatt cagcgtctgac gatatgggtga tgatgtttgt gatgctgctc atcgtctcag
1921 ccgtggctgt ctttctcttt gactacttca gccctgtggg ttataacagg tgccctcgctg
1981 atggcagaga gccgtgtgga cctctcttca ccacggcaa agctatttgg ttgctctggg
2041 gtctggtgtt taacaactcc gtacctgtgc agaaccctaaa ggggaccacc tccaagatca
2101 tgggtgtcagt gtgggccttc tttgctgtca tcttctggc cagctacact gccaaacttag
2161 ctgccttcat gatccaagag gaatatgtgg accaggtttc tggcctgagc gacaaaaagt
2221 tccagagacc taatgacttc tcacccctt tccgctttgg gaccgtgccc aacggcagca
2281 cagagagaaa tattcgcaat aactatgcag aaatgcatgc ctacatggga aagttaacc
2341 agaggggtgt agatgatgca ttgctctccc tgaacacagg gaaactggat gccttcatct
2401 atgatgcagc agtgcgtgaac tatatggcag gcagagatga aggtgcaag ctggtgacca
2461 ttggcagtgg gaaggtcttt gcttccactg gctatggcat tgccatccaa aaagattctg
2521 ggtggaagcg ccagggtggac cttgctatcc tgcagctctt tggagatggg gagatggaag
2581 aactggaagc tctctggctc actggcattt gtcacaatga gaagaatgag gtcattgagca
2641 gccagctgga cattgacaac atggcagggg tcttctacat gttggggggcg gccatggctc
2701 tcagcctcat caccttcac tcgcaacacc tttctattg cgacttccga cattgtctta
2761 tgggtgtctg ttctggcaag cctggcatgg tcttctccat cagcagaggt atctacagct
2821 gcattccatgg ggtggcgatc gaggagcgcc agtctgtaat gaactcccc accgcaacca
2881 tgaacaacac acactccaac atcctgcgcc tgctgcgcac ggccaagaac atggctaacc
2941 tgtctggtgt gaatggctca ccgcagagcg ccttggaact catccgacgg gagtcatccg
3001 tctatgacat ctacagagc cgcgcagct tcacgcattc tgactgcaaa tctacaaca
3061 acccgccctg tgaggagaac ctcttcagt actacatcag tgaggtagag agaacttgc
3121 ggaacctgca gctgaaggac agcaacgtgt accaagatca ctaccacat caccaccggc
3181 cccatagtat tggcagtgcc agctccatcg atgggtctc cgactgtgac aaccacacct
3241 tcaccacca gtccaggtcc atcagcaaga agccccctgga catcggcctc cctctctca
3301 agcacagcca gctcagtgc ctgtacggca aattctctt caagagcgac cgctacagt
3361 gccacgacga ctgatccgc tccgatgtct ctgacatctc aaccacacc gtcacctatg
3421 ggaacatcga gggcaatgcc gccaaaggc gtaagcagca atataaggac agcctgaaga
3481 agcggcctgc ctggccaag tccgcaggg agtttgacga gatcgagctg gcctaccgtc
3541 gccgaccgcc ccgtccccct gaccacaagc gctacttcag ggacaaggaa gggctacggg
3601 acttctacct ggaccagtc cgaacaaagg agaactcacc cactgggag cacgtagacc
3661 tgaccgacat ctacaaggag cggagtgatg actttaagcg cgactccatc agcggaggag
3721 ggccctgtac caacaggtct cacatcaagc acgggacggg cgacaaacac ggcgttgta
3781 gcgggggtacc tgcacctgg gagaagaacc tgaccaactg ggagtgggag gaccggtccg

3841 ggggcaactt ctgccgcagc tgctcccca agctgcacaa ctactccacg acggtgacgg
 3901 gtcagaactc gggcaggcag gcgtgcatcc ggtgtgaggc ttgaagaaa gcaggcaacc
 3961 tgtatgacat cagtgaggac aactccctgc aggaactgga ccagccggct gccccagtgg
 4021 cggtgacgtc aaacgcctcc accactaagt accctcagag cccgactaat tccaaggccc
 4081 agaagaagaa ccggaacaaa ctgcgccggc agcactccta cgacaccttc gtggacctgc
 4141 agaaggaaga agccgacctg gccccgcgca gcgtaagcct gaaagacaag ggccgattca
 4201 tggatgggag cccctacgcc cacatgtttg agatgtcagc tggcgagagc acctttgcca
 4261 acaacaagtc ctcaagtccc actgccggac atcaccacca caacaacccc ggccggcgggt
 4321 acatgctcag caagtcgctc taccctgacc gggtcacgca aaacctttc atccccactt
 4381 ttggggacga ccagtgttg ctccatggca gcaaatccta cttcttcagg cagcccacgg
 4441 tggcgggggc gtcgaaagcc aggcgggact tccggggcct tgcaccaac aagccggtgg
 4501 tctcggccct tcatggggcc gtgccagccc gttccagaa ggacatctgt atagggaacc
 4561 agtccaaccc ctgtgtgctt aacaacaaaa accccagggc tttaaatggc tccagcaatg
 4621 ggcatgttta tgagaaactt tctagtattg agtctgatgt ctgagtgagg gaacagagag
 4681 gttaaggtgg gtacgggagg gtaaggctgt gggtcgcgtg atgcgcatgt cacggagggt
 4741 gacgggggtg aacttggttc ccatttgctc ctttctgtt ttaattatt tatgggatcc
 4801 tggagttctg gttcctactg ggggcaaccc tggtgaccag caccatctct cctcctttc
 4861 acagttctct ctttctccc cccgctgtca gccattctg tcccatgag atgatgcat
 4921 gggccctctc agcaggggag ggtagagcgg agaaaggaag ggctgcatgc gggtctcctc
 4981 ctggtgtgga agagctcctt gatatcctct ttgagtgaag ctgggagaa caaaaagagg
 5041 ctatgtgagc acaaaggtag ctttcccaa actgatcttt tcatttaggt gaggaagcaa
 5101 aagcatctat gtgagacat ttagcacact gcttgtgaaa ggaaagaggc tctggctaaa
 5161 tcatgctgc ttgatgaca tctgtctagg aatcatgtgc caagcagagg ttgggaggcc
 5221 atttgtgtt atatataagc ccaaaaatgc ttgcttaac ccatgagac tcgatagtgg
 5281 tggagaacag aaccaaggt cattggtggc agagtggatt ctgaacaaa ctggaaagta
 5341 cgttatgata gtgtccccg gtgccttggg gacaagagca ggtggattgt gcgtgcatgt
 5401 gtgtcatgc acactgcac ccatgttag tcaggtgcct caagagaagg caacctgac
 5461 tcttctatt gtttcttca atatcccaa gcagtgtgat tgttggtt atatacagac
 5521 agataggcc atgtattacc tgaatttg ctgtgtctc cttcatcct ctggaataag
 5581 gagaatgaaa attcttgata aagaagattc tgtggtctaa aaaaaaaag gcggtgagca
 5641 atcctgcaag aacaaggtag ataacaagt cctcagtggg tggcaattgt ttcaaccagt
 5701 ttgaaccaag aacttccag gaaggctaaa gggaaaccga atttcacag ccatgattct
 5761 ttgcccaca cttgggagca aaagattcta caaagctctt ttgagcattt agactctega

5821 ctggccaagg ttggggaag aacgaagcca ctttgaaga agtaaggagt cgtgtatggt
 5881 agggtaagt agagaggggg atgttccaa tgcttgatc ctttctact taacctgaag
 5941 ctagacgagc aggtctctc ccccaaac tgattacaac tgctacagag cagacagtta
 6001 agagaaatga gcttgacctt taagagaaat gagctgcact ccatgagtgc agctctggag
 6061 gtacgaaaag aggggaagag acttggaat gggagacggg ggcagagagg gacctccac
 6121 cactctttg ggctggctc cctgggaatg tgactgagc ccagagtga cactcttgg
 6181 agaagccctt ctacctct gcaacacctt gttccctct cagattgtac cattgag

SEQ ID NO:15 shows a 60 oligonucleotide fragment target, as follows:

SEQ ID NO:15

g gttacatcaa aaaatgctgc aaggggttct gtattgacat ccttaagaaa atttctaaa

SEQ ID NO:16 shows one oligonucleotide primers (21 nucleotides), as follows:

SEQ ID NO:16

tcactgagaa taaaacagac g

SEQ ID NO:17 shows one oligonucleotide primers (21 nucleotides), as follows:

SEQ ID NO:17

t cacctatgac cttacctgg

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.) but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at room temperature, and pressure is at or near atmospheric.

T02030" T022660

EXAMPLE 1. PREPARATION OF POLYCLONAL ANTIBODIES (IgG) TO GLUTAMATE AND HOMOCYSTEINE.

Glutamate (polyglutamate, 10 aminoacids) or homocysteine (polyhomocysteine, 10 aminoacids) alone will not generate antibodies when injected into an animal. Therefore, polyglutamate and polyhomocysteine were conjugated with human serum albumin for the immunization to obtain polyclonal antibodies. For glutaraldehyde conjugation, polyglutamate or homocysteine (10 mg) and 40 mg bovine serum albumin (BSA, Sigma, St. Louis, MO) were incubated for 2 hr at room temperature in 4 ml of PBS containing 5% glutaraldehyde. The reaction was stopped by adding glycine to a final concentration of 0.2 M, and the conjugate was dialyzed against PBS.

Rabbits were given initial injections of 1 mg of conjugated glutamate (polyglutamate) or homocysteine (polyhomocysteine) in complete Freund's and subsequent increased doses of injections (2 mg) in incomplete Freund's adjuvant at successive 2 week intervals. All injections were given subcutaneously. The immunization period lasted for 110 days. Antibodies (IgG) were affinity purified according to standard procedures (Warr, G.W., Purification of antibodies, In: Antibody as a Tool, Eds., Marchalonis, J.J., and G.W. Warr, J. Wiley, UK, pp. 59-96 (1982)) and were shown to be selective for glutamate or homocysteine by ELISA assay.

EXAMPLE 2. PREPARATION OF POLYCLONAL ANTIBODIES (IgG) TO NR2A RECEPTOR PEPTIDE.

Using computer analysis of the hydrophobicity profile of human NR2A and NR2B NMDA receptors to predict the antigenic determinants in the protein structure, we selected fragments corresponding to the N-terminal sequence of human NR2A and NR2B receptor peptides for synthesis. The fragments corresponded to the N-terminal sequence of the NR2A and NR2B receptors, represented by SEQ ID NO: 1 and SEQ ID NO: 2 for the NR2A and NR2B receptors, respectively. The peptide fragments were reproduced using solid-phase synthesis, and had a purity ranging from 90% to 98%. The peptide sequences were verified by amino acid analysis after acid hydrolysis. A mixture of NR2A and NR2B peptides (1:1) was conjugated with human serum albumin for the immunization to obtain polyclonal antibodies. For glutaraldehyde conjugation, 10 mg of the mixture of peptides and 40 mg human serum albumin (Sigma, St. Louis, MO) were incubated for 1.45 hr at room temperature in 4 ml of PBS containing 5% glutaraldehyde. The reaction was stopped

by adding glycine to a final concentration of 0.2 M, and the conjugate was dialyzed against PBS.

Rabbit polyclonal antibodies were raised against the NR2A-B peptides. Rabbits were given initial injections of 1 mg of conjugated peptides in complete Freund's adjuvant and subsequent injections (0.5 mg) in incomplete Freund's adjuvant at successive 2 week intervals. Antibodies were affinity purified according to standard procedures (Warr, G.W., Purification of antibodies, In: Antibody as a Tool, Eds., Marchalonis, J.J., and G.W. Warr, J. Wiley, UK, pp. 59-96 (1982)) and were shown to be selective for NR2A and NR2B NMDA receptors using an ELISA assay.

EXAMPLE 3. PREPARATION OF LATEX BEADS CONTAINING BIOMARKER ANTIBODIES.

Three different sensitized latex beads containing IgG against glutamate, homocysteine and NR2A-B receptor peptides were prepared using two types of blue polystyrene latex beads (diameter, 0.25 and 0.4 μM ; Sigma, St.Louis, Mo.) as follows. A 1% suspension of latex beads in 50 mM PBS (1 ml, pH 7.0) was mixed with an equal volume of corresponding IgG (2 mg/ml) and incubated on a shaker at room temperature for 2 hours. The mixture was then washed twice with PBS by centrifugation at 9,500 g for 5 min. The pellet was suspended in PBS containing 1 % BSA overnight at 4 °C. After being washed twice with PBS, the sensitized latex beads were resuspended in latex diluent (50 mM PBS with 1 % BSA) at a concentration of 0.4 % and stored at 4 °C until used.

Preliminary experiments with latex agglutination (LA) alone were performed to identify problems and to select the most desirable latex particle size. Two types of commercial latex beads were coated with antibodies at various concentrations. Tests were initially performed with the corresponding aminoacid or NR2A and NR2B receptor peptides as controls. Particle size and IgG concentration were found to be the primary factors affect the sensitivity of the test. The most desirable particle size was found to be 0.25 μm (blue latex) because particles of this size agglutinated each aminoacid and peptide specifically. Higher IgG concentrations showed higher sensitivities. Using blue latex bead coated with 2 mg of IgG per ml, agglutination could be observed within 30 min.

EXAMPLE 4. LATEX AGGLUTINATION ANALYSIS OF BLOOD SERUM SPECIMENS

Blood samples (5 ml) were collected using standard venipuncture clinical protocol, from patients with TIA, stroke and brain injury (n = 30) and examined at the laboratory of

CIS Biotech, Inc. in Atlanta (GA, USA). None of the patients had been treated with anticoagulants, and serum samples were obtained from the clotted blood. All specimens were free of visible lipids, white blood cells, platelets, fibrin, mucus or other contaminants that could cause “false positive” reactions. Platelets, white blood cells, mucus and fibrin were removed by centrifugation. Lipids were removed by filtration.

Specimens to be tested within 72 hours after collection were stored at 2 - 8 °C. For longer storage periods, - 20 °C or colder is recommended.

The semi-quantitative analysis of glutamate, homocysteine and NR2A-B receptor peptides in the serum samples is basically a three step process: serum sample dilution, reaction of latex beads with serum samples, and product analysis.

In previous experiments serial dilutions of the serum samples from 1:4 to 1:64 in saline containing 4 % glycerol for better agglutination were performed. The highest dilution in which agglutination was observed corresponded to the sample titer.

Two 25 µl aliquots of coated latex beads containing the corresponding IgG were layered on a double-concave slide (Fisher Sci., Norcross, GA), one with 25 µl of the serum sample in serial dilution to be tested and one with 25 µl of PBS as a negative control. After gentle mixing with vortex, agglutination was judged macroscopically against a dark background. A negative reaction corresponded to a homogeneous lactescent background with no agglutination; a positive reaction corresponded to a clearly visible agglutination against the black background and weakly visible agglutination on a slightly lactescent background.

The highest dilution at which agglutination occurs gives the titer of the sample. To obtain the approximate titer in µg/ml we used the following calculation:

$$\text{Titer } \mu\text{g/ml} = A \times D$$

where A is the test sensitivity, and D is the highest dilution at which agglutination occurs.

EXAMPLE 5. DESCRIPTION OF PATIENTS

Patients observed in trials (n=68) included 9 with pre-stroke, 9 with TIA (mean age 52.0 ± 3.0), 31 with acute ischemic stroke (mean age 54.7 ± 1.4) and 11 with mild brain injury (mean age 53.0 ± 4.4). Clinical evaluation of patients by neuroimaging (CT, MRI, arteriography, Doppler ultrasonography, EEG), detailed physical and neurologic examination and laboratory tests was performed. Patients with TIA were characterized by

contra lateral weakness, dysphasia, transient blurring of vision or blindness, abnormal pulsation of the common carotid arteries, microemboli confined to the ipsilateral retina. Untreated patients with pre-stroke demonstrated altered state of consciousness, severe headache, nausea and vomiting, visual disturbances, and focal neurological deficit, with some patients experiencing seizures.

The N-Score rating scale reported in "MCA Infarction" (Orgogozo, 1986) was used for evaluating the neurologic deficit in patients with acute cerebral stroke. The total score of acute cerebral stroke clinical manifestation differentiated severe patients (n=9, 11-35 scores) from patients with mild (n=12, 36-55 scores) and moderate patients (n=10, 60-90 scores). Most patients with acute cerebral ischaemia (61.3%) suffered ischemia in the carotid artery of left hemisphere. Arterial hypertension and cerebral atherosclerosis etiologically corresponded in all patients.

The patients with ischemia were divided into groups based on the differences between TIA, pre-stroke and acute ischemic pathogenic mechanisms. The clinical diagnosis was established on the basis of routine observations which included detailed neurological examination and neuroimaging. Groups of TIA (n=9) and pre-stroke patients with chronic cerebral blood insufficiency (n=9) were identified by neurophysiological investigations.

EXAMPLE 6. DETECTION OF GLUTAMATE AND HOMOCYSTEINE IN THE BLOOD OF PATIENTS

Glutamate and homocysteine content were measured by standard high performance liquid chromatography (HPLC) according to methods described (Perry I.J., Refsum H., Morris R.W., Ebrahim S.B., Ueland P.M., Shaper A.G. *Lancet*. 1995, 346:1395-1398; Yamamoto T., Rossi S., Stiefel M., Doppenberg E., Zauner A., Bullock R., Marmarou A. *Acta Neurochir.Suppl.* 1999, 75: 17-19). The limits of the normal range were 165.0 $\mu\text{mol/L}$ for glutamate (Table 1) and 8.0 $\mu\text{mol/L}$ for homocysteine (Table 2). Elevated glutamate and homocysteine amounts were detected in the blood of patients with acute stroke. However, approximately 66 % of these patients had additional risk factors indicative of atherosclerotic processes such as high cholesterol and LDL levels (Denisenko T.V., Skuliabin D., Gromov I., Cherkas Yi., Iluchina A., Dambinova S.A., 1998. *Vopr. Med. Khimii*. 44, 584-590, in Russian).

Abnormal glutamate and homocysteine plasma concentrations were observed more frequently in patients with TIA than in patients with acute stroke. The positive predictive

efficiency of plasma glutamate for TIA patients was 56 %. The positive predictive efficiency of plasma homocysteine for TIA patients was 66%. Baseline concentrations for glutamate and homocysteine are 160 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$, respectively. Routine treatment for TIA was found to consistently decrease the glutamate and homocysteine levels in the blood of patients (data not shown).

In patients with pre-stroke, slightly elevated levels homocysteine were observed; levels of glutamate were unchanged (Tables 1, 2). In patients with traumatic brain injury (TBI), glutamate levels were observed that were nearly twice the glutamate levels in healthy individuals; levels of homocysteine were up to 57 % higher.

Table 1
Glutamate concentration in the blood of patients detected by HPLC

Group	Total N	Glutamate $\mu\text{mol/L}$	HPLC predictive value			
			Negative		Positive	
			N	%	N	%
Healthy individuals	28	165.0 \pm 28.2	19	67.8	9	32.2
TIA	9	200.0 \pm 11.7	4	44.4	5	55.6
Pre-stroke	9	163.7 \pm 10.4	5	55.6	4	44.4
Acute stroke	31	172.1 \pm 20.6	13	41.9	18	58.1
TBI	11	305.0 \pm 28.8	4	36.4	7	63.6

We also compared homocysteine concentrations in the blood of patients with TIA and pre-stroke to homocysteine concentrations in the blood of patients who have had stroke onsets. We observed that homocysteine content in the blood of patients depended on stage of the stroke, but that homocysteine concentration did not correlate with the severity of the cerebral ischemia. A significant decrease in homocysteine levels in patients with acute stroke was observed after emergency therapy (data not shown).

Latex agglutination was also employed to detect TIA/stroke biomarkers in the blood serum of patients. The titer of plasma glutamate determined by latex agglutination was 3.34 \pm 0.25 in the group of healthy volunteers. Homocysteine and glutamate trends observed using HPLC were similarly observed for different groups of patients observed by using the LA technique (Tables 3, 4). Thus, increased levels of glutamate and homocysteine were similarly observed in the blood of patients with TIA and acute stroke using LA.

With respect to predictive efficiency, however, LA showed a surprising improvement over HPLC. For example, the LA method improved the positive predictive efficiency of patients with TIA and acute stroke on the basis of glutamate content to more than 63 % (Tables 1, 3). The negative predictive value for healthy patients was similarly improved when using the LA technique (Tables 3, 4). The predictive value of the LA technique in the group of patients with TBI was identical to the predictive value using HPLC.

Table 2

Homocysteine concentration in the blood of patients detected by HPLC

Group	Total N	Homocysteine $\mu\text{mol/L}$	HPLC predictive value			
			Negative		Positive	
			N	%	N	%
Healthy individuals	28	8.0 ± 1.7	20	71.4	8	28.6
TIA	9	10.8 ± 1.3	3	33.3	6	66.4
Pre-stroke	9	9.0 ± 1.2	4	44.4	5	55.6
Acute stroke	31	11.5 ± 1.1	11	35.5	20	64.5
TBI	11	12.6 ± 2.1	4	36.4	7	63.6

Table 3.

Detection of glutamate in the blood of patients by latex agglutination

Group	Total N	Glutamate Titer	LA predictive value			
			Negative		Positive	
			N	%	N	%
Healthy individuals	28	3.34 ± 0.25	22	78.6	6	21.4
TIA	9	4.52 ± 0.38	3	33.3	6	66.4
Pre-stroke	9	3.57 ± 0.32	4	44.4	5	55.6
Acute stroke	31	4.34 ± 0.47	11	35.5	20	64.5
TBI	11	5.12 ± 0.62	4	36.4	7	63.6

Table 4.

Detection of homocysteine the blood of patients by latex agglutination

Group	Total N	Homocysteine Titer	LA predictive value			
			Negative		Positive	
			N	%	N	%
Healthy individuals	28	2.23 ± 0.21	21	75.0	7	25.0
TIA	9	3.95 ± 0.37	3	33.3	6	66.4
Pre-stroke	9	2.89 ± 0.12	4	44.4	5	55.6
Acute stroke	31	4.01 ± 0.41	10	32.3	21	67.7
TBI	11	4.74 ± 0.38	4	36.4	7	63.6

EXAMPLE 7. THE DETECTION OF NR2A-B IN THE BLOOD OF PATIENTS

The excessive activation and damage of NMDA receptors is the result of glutamate, aspartate and homocysteine neurotoxicity. Autoantibodies to have been detected in previous work in the blood of patients with TIA and pre-stroke, supporting our hypothesis that cerebral ischemia causes neuronal damage and the appearance of autoantibodies to NMDA receptor subunits (Gusev E.I., Skvortsova V.I., Alekseev A.A., Izykenova G.A., Dambinova S.A. S.S Korsakov's J.Neurol.& Psych. 1996, 5:68-72; Dambinova S.A., Izykenova G.A. J.High Nervous Activity. 1997, 47: 439-446).

The titer of NR2A-B receptor peptides in the blood of healthy volunteers determined by LA was 2.63±0.92. Using the LA technique, we observed an increase in the test efficiency in the group of healthy persons up to 89 % (Table 5). We also observed an improvement in the sensitivity of the LA test over ELISA. For example, patients with pre-stroke had slightly increased levels of NR2A-B receptor peptides over healthy volunteers when tested by ELISA, but had nearly double the level of NR2A-B receptor peptides when measured by LA (Table 5, 6). We detected high levels of NR2A-B receptor peptides using both ELISA and LA in the blood of patients with TIA and acute stroke, and observed comparable levels of predictive efficiency for each test.

Patients with TIA received routine treatment to improve brain circulation. Upon receiving treatment, NR2A-B levels decreased to levels corresponding to those observed for the healthy individuals as the patient's state normalized. As mentioned earlier, glutamate and homocysteine contents also decreased during treatment, but it never reached the levels observed in healthy individuals.

Table 5.

Detection of NR2A-B receptor peptides in the blood of patients by latex agglutination

Group	Total N	NR2A-B Titer	LA predictive value	
			N	%

			Negative		Positive	
			N	%	N	%
Healthy individuals	28	2.63±0.92	25	89.3	3	10.7
TIA	9	7.34±0.43	2	22.2	7	77.8
Pre-stroke	9	4.21±0.26	2	22.2	7	77.8
Acute stroke	31	5.20±1.71	4	9.7	27	87.1
TBI	11	3.99±0.44	2	18.8	9	81.8

Completely different profiles of NR2A-B were revealed in the blood of patients with acute ischemic stroke. In the blood of patients (n=8) with severe cerebral ischaemia (30.4 ± 3.2 Orgogozo scores) NR2A-B receptor peptides titer was 4 times higher than that for control group of healthy individuals. The peptides titer for patients with mild to moderate ischemic stroke (n=22, 49-62 Orgogozo scores) was slightly elevated in comparison with those with TIA. The tendency of slight decreases in NR2A-B receptor peptide levels was observed to the end of 30 days of patients' routine treatment, correlating with improvement in the neurological state.

Table 6

Detection of NR2A-B receptor peptides in the blood of patients by ELISA

Group	Total N	NR2A-B Ng/ml	ELISA assay results			
			Negative		Positive	
			N	%	N	%
Healthy individuals	28	18.2 ± 2.1	20	71.4	8	28.6
TIA	9	66.6 ± 4.1	2	22.2	7	77.8
Pre-stroke	9	23.7 ± 1.9	3	33.3	6	66.7
Acute stroke	31	73.4 ± 6.5	5	16.1	26	83.9
TBI	11	54.3 ± 4.9	3	27.3	8	72.7

It is necessary notice that efficiency of both laboratory assays to detect the NR2A-B receptor peptides in the blood of patients with TIA/stroke and traumatic brain injury have been determined as 78 and 82 % correspondingly.

The simultaneous detection of all brain damage biomarkers: glutamate, homocysteine and NR2A-B receptor peptides in the blood patients by latex agglutination allowed to diagnose rapidly TIA/stroke with efficiency up to 85- 89%. The simultaneously increased levels of all biomarkers in the blood reflect the neurological deficit and may be

Department of Neurology of Human Brain Institute, St. Petersburg Russia. The blood specimens were used for total DNA isolation or applied on FTA paper circles.

The quantitative analysis of NR2A cDNA expression in the serum samples is basically a three step process: Total DNA isolation and purification from sera of individuals; specific cDNA coding NR2A receptor amplification; and product analysis.

The total DNA isolated by DNAzol (Mol.Res.Center, Inc., Cincinnati, OH) or bound on FTA blood staining collection cards (Life Technologies, Inc., Gaithersburg, MD) serves as a template for the polymerase chain reaction (PCR). In the first variant, the PCR assay uses a set of specially designed primers (50 pmol), immobilized on solid matrix of microplates and amplifies a specific cDNA sequence (620 bp) coding the NR2A glutamate receptor. In a second variant, the PCR assay uses a master ready-to-use buffer and amplifies cDNA bound on FTA paper. Following amplification, the quantity of a product is determined by enzyme or non-enzyme color reaction with a substrate.

Using the DNAzol reagent for DNA isolation, the whole blood of each individual (0.5 ml) was combined with 1 ml DNAzol (Mol.Res.Center, Inc., Cincinnati, OH) for 5 min at room temperature and lysed (Mackey K. et al. Mol.Biotechnol. 9: 1-5 (1997)). The organic phase (0.4 ml) of each sample was transferred to a clean tube and 0.4 ml isopropanol was added. The mixture was incubated for 5 min at room temperature and centrifugated at 6,000 g for 6 minutes. The pellet was washed in 0.5 ml DNAzol and centrifugated at the same conditions. The total DNA pellet was mixed with 1 ml of 75 % ethanol and centrifuged at 6,000 g for 5 minutes. Then the DNA pellet was diluted in 200 μ l of 8 mM NaOH and incubated at room temperature for 5 min followed by vortexing. Alkaline DNA solution was then neutralized with 160 μ l of 0.1 M HEPES, pH 7.4.

Immobilization of oligonucleotide probes (primers, SEQ ID NO:8) was performed as follows. A total of 100 μ l of 3x PBS buffer containing the primers (150 nM) was dropped into each well of a 96-well microtiter plate (Fisher Sci., Suwanee, GA). After incubation for 2 h at 37 °C or overnight at room temperature, the plate was washed three times with 1x PBS buffer containing 0.05 % (w/vol) Tween 20. The oligonucleotide-coated plates were stable for 2 months at 4°C.

Direct PCR reactions were performed in a final volume of 50 μ l (Sisk RB. in book: Molecular diagnostics: for the clinical laboratorian. Ed. by Coleman WB., and Tsongalis GJ. Humana Press Inc., Totowa, NJ. 1997, pp.103-121). The total DNA (5 μ l), isolated from blood samples of individuals, to oligonucleotide-coated plate in duplicates and 45 μ l of

master ready-to-use buffer containing 1 l TaKaRa Z-Taq DNA polymerase (TaKaRa Biomedicals, Otsu, Shiga, Japan) 10 l AMV/Tfl 5X reaction buffer, 1 l dNTP mix (Promega, Madison, WI) 2 l of 25mM MgSO₄ were added and sealed. The 30-thermal cycles (98°C - 5 s, 66°C - 2 sec) amplification using programmable Gene Cycler thermocycler (Bio-Rad Lab., Hempstead, UK) for 20 minutes was performed. Then 50 l of PicoGreen reagent (Mol.Probes, Inc., Eugene, OR) were added to each PCR products and mixed on a shaker (BioTechniques 20:676 (1996) . Samples were incubated 5 min at room temperature, protected from light. After incubation the fluorescence of the samples was measured using a fluorescence microplate reader (Mol.Device, Sunnyvale, CA) and standard fluorescein wavelengths (excitation 480 nm, emission 520 nm). The fluorescence value of the reagent blank was subtracted from that of each of the samples, and the data was employed to generate five-point standardization curves of fluorescence versus DNA concentration, from 25 pg/ml to 25 ng/ml reaction of control target cDNA (50 ng/ml stock) with the same Pico Green reagent.

The other method of total DNA isolation is follows. Whole blood was spotted onto FTA paper and lysed, and samples of DNA immobilized within the matrix of the stain card were punched into a 3mm (1/8") diameter paper (1mm or 2mm Harris Micro-Punch™) and amplified directly by the amplification mix (Mackey K. et al. Mol.Biotechnol. 9: 1-5 (1997).

The FTA Bloodstain Card is divided into 4 circles for at least 4 different 120 l samples of EDTA collected whole blood. Samples of blood were dried at room temperature for at least 1 hour. A circle was drawn with a #2 pencil around each blood to visualize where the blood had been spotted after the FTA paper processing. The FTA Bloodstain Card was then placed in a small plastic tray and 50 ml of FTA Purification Reagent was added and incubated on a shaker for 5 minutes. FTA Purification Reagent was replace 3 times with 25-50ml of the fresh solution and shaken for an additional 5 minutes. Then 25-50ml of TE-4 (10mM Tris-HCl pH 8.0; 0.1mM EDTA pH 8.0) was added and the mixture incubated twice on a shaker for 5 minutes. The FTA Bloodstain Card was allowed to air dry completely during 2 hours at room temperature. The samples were then punched from the cards using a 3mm diameter punch or the Harris Micro-Punch (1.2mm or 2.0mm), and transferred into corresponding microplate wells. PCR was then performed using the above-described procedure using regular PCR microplates and a ready-to-use buffer containing primers.

Patients (n=30, the age of 44-77) were divided into two groups. The first group of patients (n=12) were diagnosed with TIA in the carotid circulatory system, according to the following neurological criteria. Neural dysfunction was localized to a specific vascular distribution; the duration of the attack was usually less than 15 minutes and never exceeded 24 hours; and the patients did not have abnormal neurologic signs between attacks. The second pre-stroke group (n=18) were diagnosed with TIA in the vertebral-basilar circulatory system. The second group of patients was subdivided on the basis of compensation or non-compensation of neurological deficit. The third group (n=12) included patients with migraine and epilepsy.

The control group of healthy individuals (n=20) showed a level of NR2A cDNA expression of 1.2 ± 0.11 pg/ml. The first group demonstrated slightly elevated levels of NR2A cDNA expression of 1.7 ± 0.13 pg/ml. The patients with compensation of neurological deficit from the second group showed a level of NR2A cDNA expression of 1.8 ± 1.4 pg/ml. At the same time, the patients without compensation of neurological deficit that possessed more severe symptoms of TIA showed levels of NR2A cDNA expression of 3 times the levels seen in healthy individuals. Patients suffering migraine and epilepsy did not show any increase of NR2A cDNA expression when compared with the control group.

EXAMPLE 10: IMMUNOLOGICAL ANALYSIS OF BLOOD SERUM SPECIMENS

Blood samples (10 ml, collected by venipuncture) from patients with cerebral ischemia (n = 70), and healthy individuals (n=200), collected according to standard clinical protocols, were examined at the Neurology Hospital of Russian Medical Academy (Moscow, Russia). The blood specimens were centrifugated (4000 g, 5 min, +4 °C) and the collected serum stored at -70°C for further analysis.

Computer analysis was employed to predict the antigenic determinants in the NR2A receptor protein structure based on hydrophobicity profile (Hopp, T.P. and K.R.Woods, *Proc. Natl. Acad. Sci. USA* 6:3824-3828 (1981)) and antigenicity (Welling, G.W., *et al.*, *FEBS Lett.* 188:215-218 (1985)). Based upon this analysis, the N-terminal sequence of the NR2A NMDA receptor was synthesized. This synthetic peptide, which corresponded to amino acid sequence (494-514) (Grandy, DK., *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 86:9762-9766(1989) (SEQ ID NO:3) of human NR2A NMDA, was produced by solid-phase synthesis in a NPS-400 semi-automated synthesizer (Neosystem Lab, France) on MBHA resin using the BOC/Bzl strategy for the first two amino acids. The peptides were purified

by preparative HPLC on a DELTAPAC™C18 column (Waters Chromatography, Milford, MA) in a H₂O/acetonitrile/0.015 TFA system. The purity of the peptides was determined by analytical HPLC and ranged from 90% to 98%. The peptide sequence was verified by amino acid analysis after acid hydrolysis. This peptide was used in immunoassays of blood serum from patients and healthy individuals.

A quantitative analysis of the level of NR2A autoantibodies in serum samples was performed by enzyme-linked immunosorbent assay (ELISA) (Ngo, T.T. and H.M. Lenhoff, *FEBS Lett.* 116:285-288 (1980)). The diluted blood sera (1:50) and polyclonal antibodies to the NR2A peptide as a standard (0.01 ng/ml-400 ng/ml) were applied to the immunosorbent. The plate was incubated for 1 h at 25° C and then washed by 0.05 M phosphate buffer, pH 7.4, containing 0.05% of TWEEN-20™. Rabbit antibodies to the human immunoglobulin labeled with horseradish peroxidase were added (Sigma, St. Louis, MO; 1:1000), and the plate was incubated for 1 h at 25° C. After incubation the wells were washed twice in the same buffer. The reaction was revealed by *o*-phenylenediamine in 0.05 M citrate buffer, pH 4.3 monitored at 490 nm on a microplate reader (BioRad, UK). The titer of NR2A autoantibodies in blood serum was determined by ELISA using a standard curve of the absorbance units of NR2A autoantibodies versus their concentration in a microtiter well plate.

The synthetic peptide corresponding to the NR2A NMDA glutamate receptors (3 µg) were immobilized on a nitrocellulose membrane (0.45 µm, Schleicher-Shuell, Germany) in phosphate-buffered saline (PBS), pH 7.4, then washed 2-3 times in the same buffer. Membranes with immobilized peptide were incubated with the diluted serum (1:50) of cerebral ischemia patients and other subjects for 1 h at 25° C, and then rinsed 4 times with the PBS buffer. Secondary rabbit anti-human immunoglobulins conjugated with horseradish peroxidase (Sigma, St. Louis, MO; 1:1000) were incubated with the membrane for 1 h at 25°C, then washed 4 times with PBS. The development of brown color was registered and then quantitated by densitometry.

To provide a positive control or standard, rabbit polyclonal antibodies were raised against NR2A synthetic peptide corresponding to amino acid sequence predicted from the cloned human NR2A protein (Science 256:1217-1221 (1992); SEQ ID NO:1). For glutaraldehyde conjugation, 10 mg of peptide and 40 mg of human serum albumin (Sigma, St. Louis, MO) were incubated for 1.45 h at room temperature in 4 ml of PBS containing 5% glutaraldehyde. The reaction was stopped by adding glycine to a final concentration of

0.2 M, and the conjugate was dialyzed against PBS. Rabbits were given initial injections of 1 mg of conjugated peptide in complete Freund's and subsequent injections of 0.5 mg of peptide in incomplete Freund's adjuvant at successive 2 week intervals. Antibodies were affinity purified according standard procedure (Warr, G.W., Purification of antibodies, In: *Antibody as a Tool*, Eds., Marchalonis, J.J., and G.W. Warr, J. Wiley, UK, pp. 59-96 (1982)) and were shown to be selective for the NR2A NMDA glutamate receptor using Western blot analysis.

The patients (men, n=30; women, n=40; age of 40-75) were admitted in the hospital within no more than six hours after the onset of an ischemic episode. All patients were divided into three groups according to the severity of the stroke: The first group had moderate ischemic stroke (n=25), manifested by moderate focal deficit (>60- Orgogozo scale). The second group had severe stroke (n=30), manifested by mild disorders of consciousness, severe headache, meningeal sings, and pronounced focal deficit (30-60 - Orgogozo scale). The third group had extremely severe stroke (n=15), accompanied by stupor-coma, signs of brain edema, autonomic dysfunction, and severe focal deficit (<30 - Orgogozo scale).

The level of NR2A autoantibodies was measured in the blood serum of healthy persons (n=200, age 35-75) as a control, and ranged from 0.3-1.5 ng/ml. The NR2A autoantibody level in the 55 patients of the first and second groups was significantly greater than that in the control group ($p<0.01$). Levels of NR2A autoantibodies were monitored every three hours during the first day, and then up to 5th day after stroke. The level of NR2A autoantibodies in the blood serum of patients with severe stroke was significantly higher than that in the blood serum of patients with moderate stroke, especially in the 9-12 hours after the onset of a stroke ($p<0,05$). The tendency for NR2A autoantibodies level to decrease to the control level on the first day of stroke was registered in group of patients with good neurological recovery (90,50,5 units on Orgogozo scale). It can be concluded that the dynamic changes in NR2A autoantibodies level may predict a recovery period of patients after ischemic stroke.

EXAMPLE 11: SPRIA ASSAY OF AUTOANTIBODIES

The solid-phase radioimmunoassay (SPRIA) of autoantibodies is performed as follows: a 10 % acetic acid solution is added for one minute to the Cooker microtiter microplates (available from Dynatech Co., USA) for activation, whereupon 0.1 ml of the

blood serum under analysis (diluted 1:40) is applied to the microplates and subjected to incubation for four hours at 25°C. Then the microplate are washed with a 0.14 M sodium chloride solution and 0.1 ml of a mixture of the respective fragment of the mammal's brain protein labeled by ¹²⁵I in the presence of nonlabelled one. The plates are incubated for 20 hours at 4°C. On completion of incubation, the microplates are washed with a 0.14M sodium chloride solution, after which each of the wells of the microplates is cut off and placed in gamma-counting vials.

EXAMPLE 12: ELISA ASSAY OF AUTOANTIBODIES

The enzyme-linked immunosorbent assay (ELISA) of autoantibodies is carried out as follows: the samples of the blood serum diluted 1:40 or 1:50 are applied to the respective immunosorbent. Then the plate carrying the immunosorbent is incubated for 30 min at 37°C, whereupon the wells of the plate are washed with a 0.05 M phosphate buffer, containing 0.05% of Tween-20. Rabbit antibodies to human immunoglobulin labeled with horseradish peroxidase (conjugate) are added thereto, and the plate is reincubated for 35 min at 37°C, then washed by the aforementioned buffer and distilled water. The reaction with conjugate is determined by adding chromogen, *i.e.*, orthophenylenediamine in the presence of 30 % hydrogen peroxide. The intensity of color development is evaluated by using the rider (available Multiskan microplate rider) at the 492 nm wavelength.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.